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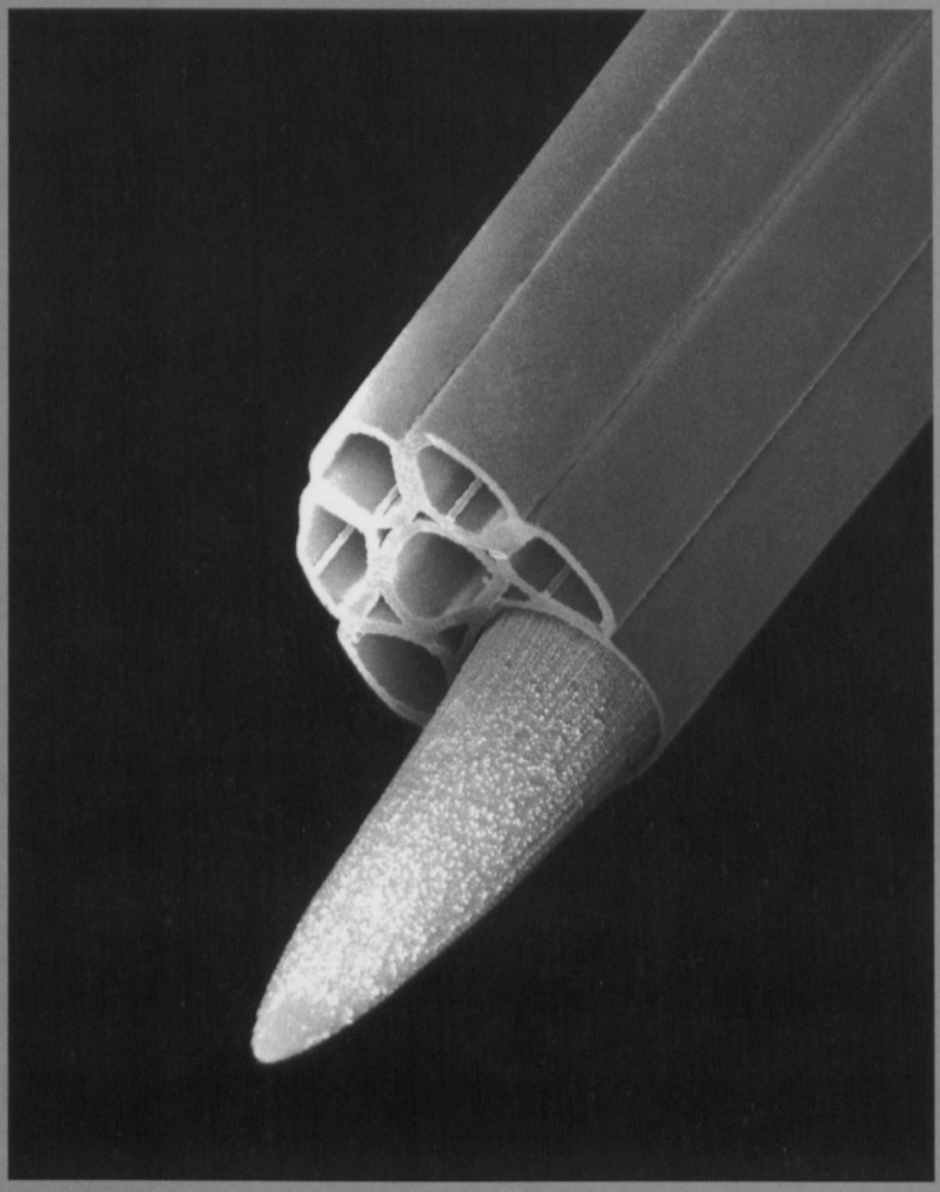

University of Szeged, Szeged, Hungary

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ARTICLE

Polymer insulation of ultramicro carbon fiber electrodes for electrophysiological, electrochemical and biosensor applications

Dénes Budai[1,2]*, Klára Horváth[1], András Szabó[1]

[1]Kation Europe Bt., Szeged, Hungary, [2]Szeged Neurobiological Knowledge Center, Szeged, Hungary

ABSTRACT There is an obvious need for electrodes with extremely small electroactive areas and structural dimensions that offer great promise for electrochemical microscopy used for neuronal analysis in ultrasmall environments. In the present study we have developed ultramicro carbon fiber (CF) electrodes with combined thin layers of poly(oxyphenylene) and epoxy resin coatings for electrical insulation. The thickness of the borosilicate glass housing and insulating layer of our standard CF microelectrodes is about 1 |im and the carbon tip protrudes by about 20 |im from the glass assembly. Functionalization requires longer sections of the exposed, uninsulated carbon fiber where submicron tips are shaped and chemical modifications are made. Electrodeposition of poly(oxyphenylene) was carried out using anodic currents at 2V against an Ag/AgCI half-cell. After finishing the polymerization at 150°C for 2 hours, electrical impedances of the electrodes were 17.1 ±2.8MQ (mean ± SD, n= 22). An additional epoxy coating was formed by dipping the CF electrodes in diluted epoxy resins followed by dipping in a mixture of diluted curing agents. The epoxy layer significantly increased the effectiveness of the insulation as the impedance for each of the 38 prepared CF electrodes was higher than the upper range limit (200 MQ) of our impedance meter. The thickness of the combined insulating layer was less than 1 |im as estimated by electron microscopic studies. Removal of the insulation from the very tip was carried out using high voltage spark or electrochemical etching. These submicron CF electrodes are suitable for extracellular spike recording, electrochemical and biosensor applications.**
Acta Biol Szeged 51(2):81-85 (2007)

KEY WORDS

carbon tip etching
scanning electron microscopy
poly(oxyphenylene)
epoxy
electrodeposition
electrical Insulation
electroactive area

Carbon fiber (CF) microelectrodes are used to record neuronal action potentials (Armstrong-James and Millar 1979; Armstrong-James et al. 1980) or to detect electrochemical signals produced by electroactive compounds such as catecolamines or nitric oxide. The carbon fibers are graphite monofilaments of about 7 pm in diameter. In microelectrodes, they have very high tensile strength, low electronic impedance and they provide oustanding extracellular recording qualities similar to those of the best tungsten electrodes (Budai and Molnár 2001; Budai 2004). By covalent modifications of their surface (Baker et al. 2005; for review see, Downard 2000), CFs are suitable for construction of biosensors on the micrometer scale (Yang 2005; Ahuja et al. 2007; Koncki 2007). In cases of these applications, the base electrode material must be electrically insulated except for a varying section of the recording or sensing tip. CF microelectrodes are usually insulated with borosilicate glass or, less frequently, applying plastic sheafing or electrodeposited polymers (El-Deen et al. 2006).

There is an obvious need for electrodes with extremely small electroactive areas and structural dimensions that offer great promise for electrochemical microscopy used for neuronal analyis in ultrasmall environments *(e.g.* single neurons or perhaps even single synapses). This requires an ultrasmall support material and an ultrathin electrical insulating layer that covers the support material except for the very tip of the electrode. In an ideal case, the insulating layer is very thin (less than 1 pm), covalently bound to the carbon surface, it has zero electrical conductivity and the exposed (uninsulated) length of the very tip can be reliably controlled. In the present study we have developed ultramicro CF electrodes with electrodeposited poly(oxyphenylene) insulating layer that are suitable for extracellular spike recording, electrochemical or biosensor applications. The electrical insulation of the submicron CF electrodes was further improved by forming additional layer of epoxy resin.

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Corresponding author. E-mail: kations@aol.com

Materials and Methods

Manufacturing CF microelectrodes

Single-barrel CF microelectrodes were made from borosilicate glass capillary tubing (1.50 mm o.d., 0.84 mm i.d., WPI, Sarasota, FL). A 15 cm long individual carbon fiber (PAN-based, T-300, Amoco Performance Products, Chichago, IL)

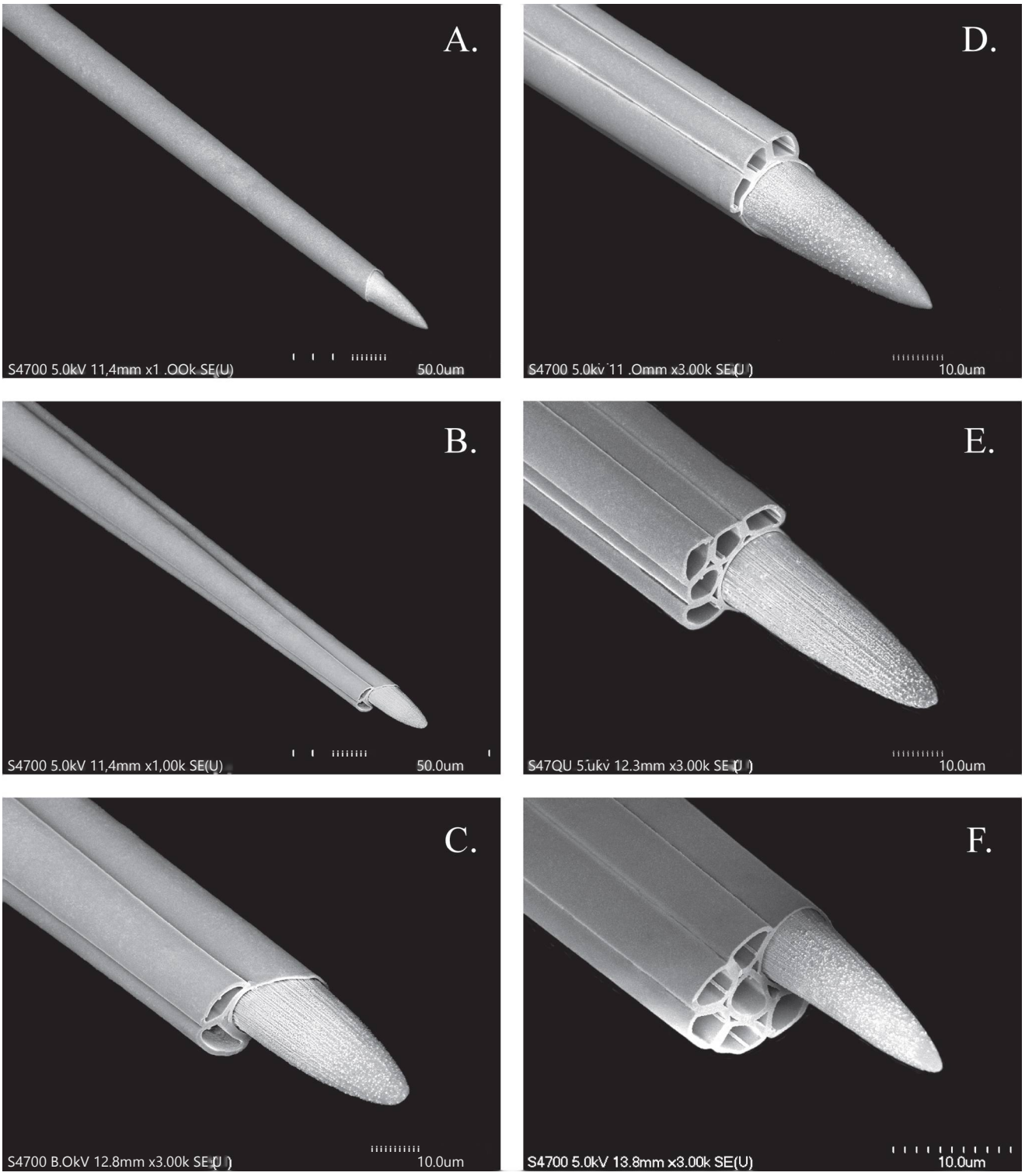

Figure 1. Scanning electron micrographs of carbon fiber microelectrodes. Single-barrel microelectrodes are consisted of a conical carbon tip protruding from the borosilicate glass insulation (A). A varying number of micropipettes can be attached to the recording carbon fiber containing barrel (B-F) for delivering drugs by microiontophoresis or pressure. Filling of the drug barrels is facilitated by inner glass microfilaments fused to the inner wall of the microcapillaries (F).

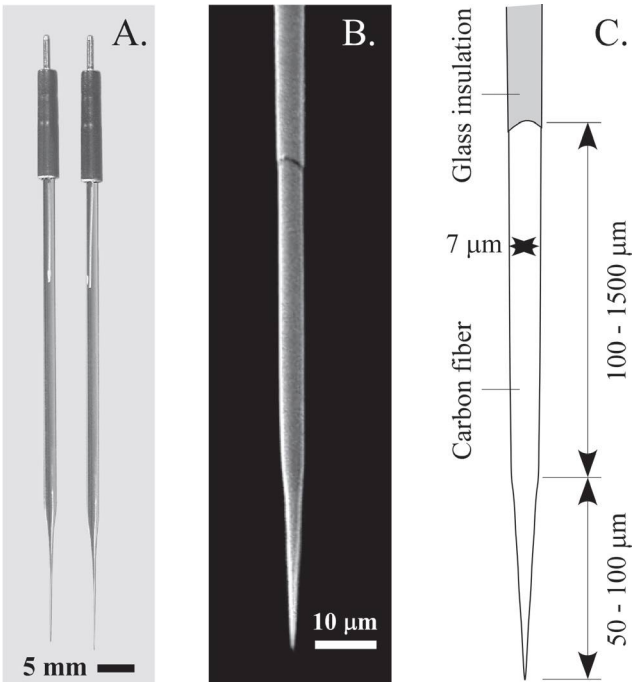

Figure 2. Macroscopic (A) and scanning electron microscopic (B) view of single-barrel carbonfiber microelectrodes made for submicron-scale electrophysiological, electrochemical or biosensor applications. Section of the carbon fiber protruding from the glass insulation (B,C) allows shaping of submicrometer tips. The exposed carbon surface can be modified chemically and is to be insulated by electropolymerization. Ultrasmall electroactive area can be formed on the very tip.

with a diameter of about 7 pm was glued to a 2.5 cm long 28 AWG tin-plated copper wire using a conductive paint. One end of the wire had previously been soldered into a gold-plated male connector pin. Beginning at its free end, the CF was sucked into the glass capillary tubing using gentle vacuum. The connector pin was then hxed onto the end of the glass tubing by 12 mm long heat-shrinkable plastic tubing. For single-barrel microelectrodes, this „blank" assembly was ready to be pulled. Construction of multibarrel CF microelectrodes was published elsewhere (Budai and Molnár 2001). The two ends of the electrode blank were then held by the chucks of a vertical micropipette puller (Gravipull-2, Kation Scientihc, Minneapolis, MN) and a heating coil was used to soften the glass gently in its central portion. Pulling force was provided with gravity only using a variable mass system. In consequence of the very high tensile strength of the CF, it did not break during the pulling procedure. The excess length of hber protruding from the tip of the glass assembly was cut with a hne pair of scissors to about 5 mm. The exposed CF was hnally trimmed to the needed length using electrochemical etching (Armstrong-James et al. 1980; Anderson and Cushman 1981) or high voltage spark etching (Budai and Molnár 2001; Millar and Pelling 2001) under a light microscope.

Chemicals and instrumentation

Polymerization solutions contained 2-allylphenol, phenol, 2-butoxyethanol, methanol and water. Curing of epoxy compounds, dodecyl/tetradecyl glycidyl ether, bisphenol A diglycidyl ether, was carried out using diethylenetriamine, 4-aminophenyl sulfone as curing agents. Methyl ethyl ketone was used for diluent. Electrochemical etching solution was made of concentrated sulfuric acid saturated with potassium bichromate. Physiological saline, 0.9% (w:v) NaCl, was used to measure impedances of CF microelectrodes. All componds were purchased from Sigma (Saint Louis, MO) and used as received.

Tip and substrate potentials were controlled by a two-electrode DC potentiostat (Micro C, WPI, Sarasota, FL) against an Ag/AgCl half cell. The applied potentials were set externally and the measured electrode currents were collected using an N16221 multifunction data acquisition board placed in a personal computer and programmed with LabView 7 (National Instruments, Austin, TX). Impedances were measured using an SC-200 impedance meter (Kation Scientihc, Minneapolis, MN) applying 1 KHz (or 110 Hz above the 20 MQ range) oscillating current. Electron microscopy was carried out using an S-4700 held emission scanning electron microscope (Hitachi, Tokyo, Japan). Electrode samples for electron microscopy were coated with conductive hlms of gold with the aid of a sputter coater model SC7620 (Quorum Technologies, East Sussex, UK).

Results and Discussion

Single- and multibarrel CF microelectrodes

Carbon hber microelectrodes made by us for extracellar spike recording and microiontophoresis are shown in Fig. 1. In its simplest form, a CF microelectrode consists of a carbon tip protruding from the borosilicate glass insulation (single-barrel CF microelectrode, Fig. 1A). View of a complete single-barrel microelectrode is shown in Fig. 2A. Electrical signals from the carbon tip leave the microelectrode through a gold-plated pin located on the top end. When extracellular spike recording is to combine with drug delivery by iontophoresis or pressure, the appropriate number of microcapillary pipettes can be attached to the recording carbon hber containing barrel (Figs. 1B-F). Conical carbon tips of these microelectrodes were formed using high voltage spark etching (Budai and Molnár, 2001). Filling of the iontophoresis barrels is facilitated by inner glass microhlaments fused to the inner wall of the microcapillaries (Fig. IF). CF microelectrodes of these types provide high quality extracellular spike recordings (Budai 2004) and allow testing drugs by iontophoretic delivery into the close vicinity of neurons (Budai et al. 1998).

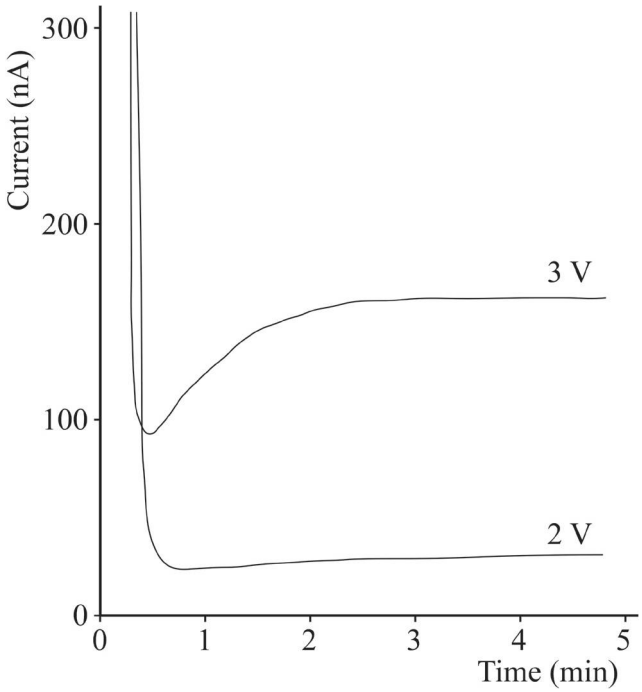

Figure 3. Current response during the electropolymerization of poly(oxyphenylene) on the carbon surface of the microelectrodes at two different holding potentials. The dip in the response at 3 V is due to a higher steady-state current eventually reached and may reflect uneven polymer formation.

Preparation of poly(oxyphenylene) insulated CF microelectrodes

The thickness of the glass insulator layer of our CF microelectrodes is about 1 pm and the carbon tip protrudes by about 20 pm from the glass assembly (Figs. 1 and 2B). Functionalization of the CF for electrochemical or biosensing applications in ultrasmall volumes requires longer sections (usually hundreds of micrometers) of the exposed, uninsulated CF where submicron tips are shaped and chemical modihcations are made (Figs. 2B,C). Following these modihcations, a new electrical insulation must be applied on the carbon surface. In our procedure to form an electrically insulating polymer, a thin layer of poly(oxyphenylene) was electrochemically deposited on the carbon surface of CF microelectrodes shown in Fig. 2B. These electrodes had carbon hbers protruding from the glass insulation by 1000 pm including a 100 pm-long section tapering into a submicron tip (Figs. 2B,C). The freshly made polymerization solution consisted of 2.6 ml 2-allylphenol, 0.53 ml phenol, 2.62 ml 2-butoxyethanol and 3.0 ml allylamine in a total volume of 100 ml of 1:1 (v:v) water-methanol mixture (Strein and Ewing 1992; Clark et al. 1997; El-Deen et al. 2006). Electrodeposition was carried out at room temperature using DC voltages in a two-electrode single-compartment electrochemical cell. An Ag/AgCl half-cell was used as reference electrode. After applying anodic current through the carbon hber electrodes using 2 V DC deposition voltage for about 5 min, the electrodes were rinsed in warm 1:1 water-methanol mixture and the polymer was cross-linked by heating at 150°C for 2 hours. Increasing the deposition voltage led to a dip in the current response due to a higher steady-state current eventually reached (Fig. 3). The higher steady-state response values may correspond with the uneven polymer formation (Strein and Ewing 1992).

The polymerization procedure consists of two steps: electrochemical generation of an electronically conducting polymer followed by curing at elevated temperature during which the deposited polymer layer becomes insulating. The o-allyl group on the monomer facilitates deposition of thicker films and also serves as a crosslink with thermal curing. After hnishing the thermal curing, impedances of the poly(oxyphenylene)-coated CF microelectrodes were measured in physiological saline using a Ag/AgCl reference electrode. An average 17.1 ± 2.8 MQ (mean ± S.D., n= 22) impedance was obtained for 22 electrodes. This value is signihcantly higher than the 0.4-0.8 MQ impedance usual for the uninsulated CF microelectrodes (Budai and Molnár 2001).

Additional insulation by epoxy resin

To improve the insulating properties of the polymer layer, an additional epoxy coating was applied. Poly(oxyphenylene) coating was electrodeposited on CF microelectrodes as described above but using 2V DC voltage for 2 min. After rinsing the electrodes in warm 1:1 (v:v) water-methanol mixture, thermal cross-linking was partially carried out for 15 min at 150°C and the electrodes were dipped in 20:1:1 (v:v:v) methyl ethyl ketone-dodecyl/tetradecyl glycidyl ether-bisphenol A diglycidyl ether epoxy mixture. Another curing period was followed at 150°C for 15 min and, lastly, the electrodes were dipped in 20:1:1 (v:v:v) methyl ethyl ketone-diethylenetriamine-4-aminophenyl sulfone mixture of curing agents. Final polymerization was carried out at 150°C for 2 hours. The additional epoxy layer signihcantly increased the effectiveness of the insulation of the CF microelectrodes as the impedance for each of the 38 prepared electrodes was higher than the upper range limit (200 MQ) of our impedance meter. The thickness of the insulating layer was less than 1 pm as estimated by light and scanning electron microscopic studies.

Re-etching of the carbon tip

Following insulation of the carbon fiber by coating with combined poly(oxyphenylene) and epxy resin, removal of the polymer from the exact tip of the electrode was accomplished in two ways. For extracellular recordings, high voltage spark etching was applied using a polished gold tip as counter electrode (Budai and Molnár 2001). We have found this method of polymer removal to give an assumedly small electroactive

area as the impedance of these electrodes averaged at 1.3 ± 0.3 MQ (mean ± SD, n= 13). For biosensing purposes, the insulating polymer needs to be removed from a longer section of the carbon tip. So, the electrodes were placed in a drop of electrochemical etching solution under a light microscope and an AC voltage of 3 to 6 V was applied for several seconds (El-Deen et al. 2006). The length of polymer removal was controlled using the hne movement of the microscope's stage. Impedances of these microelectrodes ranged from 0.6 to 1.1 MQ.

Acknowledgment

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References

Ahuja T, Mir IA, Kumar D, Rajesh (2007). Biomolecular immobilization on conducting polymers for biosensing applications. Biomaterials 28(5):791-805.

Armstrong-James M, Fox K, Millar J (1980) A method for etching the tips of carbon fibre microelectrodes. J Neurosci Methods 2:431-432.

Anderson CW, Cushman MR (1981) A simple and rapid method for making carbon fiber microelectrodes. J Neurosci Methods 4:435-436.

Armstrong-James M, Millar J (1979) Carbon fibre microelectrodes. J Neurosci Methods 1:279-287.

Baker SE, Tse KY, Hindin E, Nichols BM, Clare TL, Hamers RJ (2005) Covalent functionalization for biomolecular recognition on vertically aligned carbon nanofibers. Chem Mater 17:4971-4978.

Budai D, Harasawa I, Fields HL (1998) Midbrain periaqueductal gray (PAG) inhibits nociceptive inputs to sacral dorsal horn nociceptive neurons through alpha$_2$-adrenergic receptors. J Neurophysiol 80(5):2244-54.

Budai D, Molnar Z (2001) Novel carbon fiber microelectrodes for extracellular electrophysiology. Acta Biol Szegediensis 45:65-73.

Budai D (2004) Ultralow-noise headstage and main amplifiers for extracellular spike recording. Acta Biol Szegediensis 48:13-17.

Downard AJ (2000) Electrochemically assisted covalent modification of carbon fiber electrodes. Electroanalysis 12:1085-1096.

Clark RA, Hietpas PB, Ewing AG (1997) Electrochemical analysis in picoliter micro vials. Anal Chem 69:259-263.

El-Deen E, El-Giar M, Wipf DO (2006) Preparation of tip-protected poly(oxyphenylene) coated carbon-fiber ultramicroelectrodes. Electroanalysis 18:2281-2289.

Koncki R (2007) Recent developments in potentiometric biosensors for biomedical analysis. Anal Chim Acta 599(1):7-15.

Millar J, Williams GV (1988) Ultra low-noise silver-plated carbon fibre microelectrodes. J Neurosci Methods 25:59-62.

Millar J, Pelling CW (2001) Improved methods for construction of carbon fibre electrodes for extracellular spike recording. J Neurosci Methods 110:1-8.

Strein TG, Ewing AG (1992) Characterization of submicron-seized carbon electrodes insulated with a phenol-allyphenol copolymer. Anal Chem 64:1368-1373.

Yang T, Mao L, Okajima T, Ohsaka T (2005) A carbon fiber microelectrode-based third-generation biosensor for superoxide anion. Biosens Bioelec 21:557-564.

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ARTICLE

Examination of the human remains from the medieval cemetery of Bátmonostor-Pusztafalu in Hungary

Gyula L. Farkas[1]*, László Józsa[2], Zsolt Bereczki[1]

'Department of Anthropology, University of Szeged, Szeged, Hungary, department of Morphology, National Institute of Traumatology, Budapest, Hungary

ABSTRACT This paper summarizes the results of the physical anthropological examinations carried out on the remains of 3783 individuals from the medieval cemetery of Bátmonostor-Pusztafalu in Hungary. Distributions of sex and age at death are described along with the observations made concerning anatomical variations, developmental anomalies, pathological alterations and taxonomic features. This study is the shorter version of a more detailed paper to be published in Opuscula Hungáriáé.

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KEY WORDS

human remains
medieval age
anthropological Investigation
Hungary

As a result of the excavations carried out in 1966 and 1977-1986 by archeologists Mihály Kőhegyi (1967) and Piroska Biczó (1978-1986), 2543 graves dating back to the second half of the 14th and the 15th centuries were excavated at the site of Bátmonostor-Pusztafalu (Southern Hungary, cca. 15 km south of the town of Baja). The hndings comprise 80% of the individuals buried in the cemetery.

Materials and Methods

In 67 graves no human remains were found, but 444 other graves contained skeletal elements of several individuals. The authors examined the remains of 3783 individuals in all. The state of preservation of the skeletons greatly varies. The remains are housed at the Department of Anthropology, University of Szeged.

In case of immature individuals, age at death was determined using the method of Stloukal-Hanáková (1978), while in case of adults, the method of Nemeskéri et al. (I960) was used. Sex was determined on the basis of 24 characteristics. Measurements were carried out on the skulls and long bones employing Martin's system. Height was calculated according to Sjpvold's practice (Sjpvold 1990).

Paleodemographic analysis was based on the Acsádi-Nemeskéri method (Acsádi and Nemeskéri 1962). Paleopathological anomalies were diagnosed on the basis of morphological observations; occasionally X-ray and CT scans were taken and histological examinations were also conducted to support diagnoses. Taxonomic analysis was carried out according to Lipták's classification using the method of Farkas (Farkas 1972). To compare metric data of our series to that of other medieval cemeteries, biological distance was determined and analysed employing Penrose's method (Penrose 1954).

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♦Corresponding author. E-mail: farlgy@bio.u-szeged.hu

Results

Age at death and sex

The age at death and sex the distribution of the hndings is summarized in Table 1. The high prevalence of 0-17 year-olds is rather uncommon (1510 skeletal remains, 39.92%). 39 of these remains were fetal skeletons. In case of the remains of adults a great difference was found between the numbers of the male and female hndings (1342 male, 35.48%, and 720 female, 19.03%). On the other hand, in case of 354 hndings (9.36%) neither age nor sex could be determined.

Paleodemographic analysis

A summed up mortality table was drawn up using the method of Nemeskéri-Harsańyi-Acsadí (Nemeskerí et al I960), based on data of age at death and sex. The results show that in population of Bátmonostor life expectancy at birth of the was 30.81 years, which approximately corresponds with the demographic status of other medieval cemeteries excavated in the Carpathian Basin. Mortality manifests itself in the very low prevalence of infants, the excessive number of 5-14 year-olds and the child/adult ratio. The 2:1 ratio of men and women must be pointed out as a peculiar feature of this cemetery. A possible explanation of this phenomenon could be the fact that there was an inhrmary in Bátmonostor in the 14th-15th centuries.

Description of the measurements and indices

As far as age groups are concerned, there is no notable difference between the sexes. The same goes for sex distribution. The mean values of measurements and indices fall within

Table **1.** Distribution ofthe findings according to age at death and sex.

Age at death		Fetus	Infantia			?	Juv.	Ad.	Mat.	Sen.	?	Total	
Sex			Neo-nate	0-7yrs	8-17 yrs		18-20 yrs	21-40 yrs	41-60 yrs	61-x yrs		n	%
Male		-	-	-	-	-	38	310	595	234	165	1342	35,48
Female		-	-	-	-	-	66	199	262	127	66	720	19,03
?		39	13	584	833	41	49	8	26	5	123	1721	45,49
Total n		39	13	584	833	41	153	517	883	366	354	3783	100
				1510									
	%	1,03	0,34	15,44	22,02	1,08	4,04	13,67	23,34	9,67	9,36		
				39,92									

the range of the medium category in most cases. No striking difference was found in any age groups of the two sexes. The available data seem to indicate that we are dealing with the remains of a rather closed community here.

We also noted that deviation of the pubic angle to one side and planoccipitalia are quite common.

Anatomical variations

Anatomic variations were observed in 827 (21.86%) cases, which means that the remains of every hfth individual showed such alterations. There was no notable difference in prevalence among children and adults. Basically there is no difference between the sexes in absolute numbers either. As there are twice as many male skeletons as females, anatomic variations more often occur in women that in men.

Some rare variations were also discovered, namely: divided ala magna (Fig. 1), suture bone in sutura coronalis, conspicuously large os bregmaticum (Fig. 2), bilateral processus frontalis ossis temporalis (Fig. 3). The high prevalence of torus palatínus, also found on children's skulls, is quite uncommon.

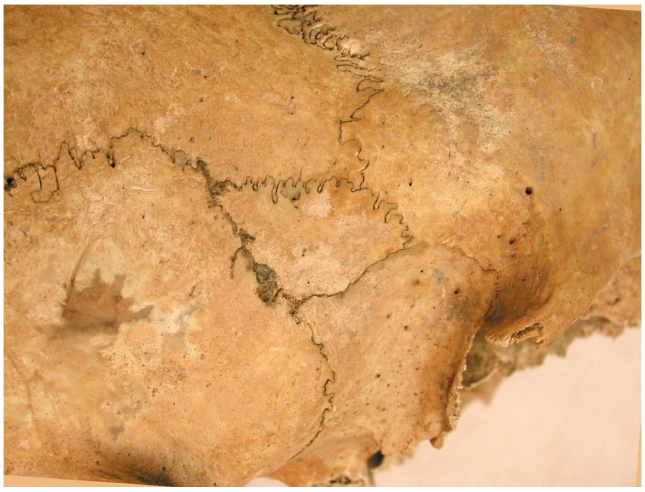

Figure 1. Grave 134: Adultus, undetermined sex, ala magna bipartitum.

The high frequency of anatomic variations also supports the concept of a closed community that we had already presumed on the basis of measurements and indices.

Developmental anomalies

Fenestration of the sternum (11), anomaly of the xyphoid process (4), syncondrosis of the sternum (7), sternum bihdum (2) and other anomalies (2) were detected in several cases, mostly in adults. The 13 cases of pectus gallinaceum are of special interest, since this anomaly is very rarely found in excavated materials (Fig. 4).

Developmental anomalies of the spine are quite common; most of them are of no consequence. Solitary sacralization was discovered in 11 adults, together with spina bihda it occurred in 15 skeletons. Solitary lumbalization was seen in one case, combined with spina bihda in four cases. Spondylolisthesis was only detected on lumbar vertebrae, in 19 cases.

Pathological alterations

Despite the state of preservation of the hndings being very versatile, quite a large number of pathological alterations could be observed on the bones. We found morphological alterations (the signs of bone diseases) on 872 skeletons. Detailed information is provided below.

Traumatic lesions

Postcranial lesion (fracture) was observed in 105 cases on 75 skeletons (79 men, 13 women, 13 individuals of undetermined sex including four children). In case of 30 skeletons, multiple fractures could be detected. Lesions affected men six times more frequently than women. Cranial lesions were found on 16 skeletons. The prevalence of bone fractures in the whole population is 2.78%, that of the skull fractures is 0.43%.

Fractures most often affected tibiae (23), ribs (22) and ulnae (19). Double fractures were observed in two cases. As for the laterality, the fractures of right/left tibiae (11/12) , radii (4:4) and ulnae (11:8) were more or less evenly distributed, while left hbulae were more often affected (4:7). Left claviculae and ribs were also more often injured. Fracture of

Figure 2. Grave 894/a: Inf. II, os bregmaticum.

the pelvis was noted in four cases.

Among the 16 skull lesions, ten affected the os frontale and four the os parietale. Skull injuries seem to be well-healed and uncomplicated, only two cases of purulent osteomyelitis were observed.

As far as other bones are concerned, healing often entailed various complications.

The prevalence of skull injuries in 10th-12th century is 30.9%, while at the site of Bátmonostor it is only 16.8%. Cranial lesions affected mostly middle-aged or elderly males, the wounds were induced by some sharp tools or weapons and the persons in question lived for a maximum of 3-4 years after the trauma.

The prevalence of fractures among the Bátmonostor findings was compared to that of a Budapest dataset from 2001 (Józsa et al. 2004). Here the fractures of the femur make up almost one fourth (23.7%) of all fractures of the lower limbs, while in the Bátmonostor series the frequency is less than 1%. The difference is similar in case of the humerus (9.0% and 1.7%). The prevalence of the fractures on the bones of the lower arms combined is basically the same in the Bátmonostor data and the recent material (2.5% and 2.2%). The isolated fracture of the ulna in the medieval series is 8 times higher (16.0%) than nowadays (2.4%). The fracture of the leg was almost twice as common in the medieval sample (9.2%) as in the modern one.

Fractures usually healed without dislocation, which indicates the presence of skilled medicine men in the population.

Figure 3. Grave 1056: Juvenis female, bilateral processus frontalis ossis temporalis.

We observed five cases of ankylosis affecting either the kneejoint (Fig. 5) or the pelvis. In the case of 3 elderly males and an elderly female, the kneejoint became ossified, the left femur of a mature male was fixed at right angles in the acetabulum. In one case evidence shows that some kind of crutch was fastened to the knee to support locomotion. Such findings are unique in the medieval material.

Non-specific infectious diseases

Osteomyelitis primarily affecting males (19 cases) was detected in 26 cases. Periostitis was recorded in 43 cases, 28 of which were males. Among the 43 cases, 17 was chronic. Ostitis was observed on the remains of 24 individuals, also affecting more males (17 cases).

The traces of non-specific infectious diseases were detected in 93 cases altogether, which makes up approximately 10% of the total number of the pathological cases. Except the 3 cases of ostitis affecting the skull, all the diseases mentioned above occurred on the limbs.

Specific infectious diseases

Pott's curvature, a typical sign of tuberculosis, along with the wedge-shaped collapse of the vertebrae was observed in three cases.

Haematological alterations

In historical anthropological samples, porotic hyperostosis can primarily be observed on the flat bones of the skull. Along with different stages of cribra orbitalia in the upper region of the orbita they both suggest iron-deficiency anaemia. Such alterations were detected in 197 cases (14.10%) within the 1397 member immature population of Batmónostor-Pusztafalu.

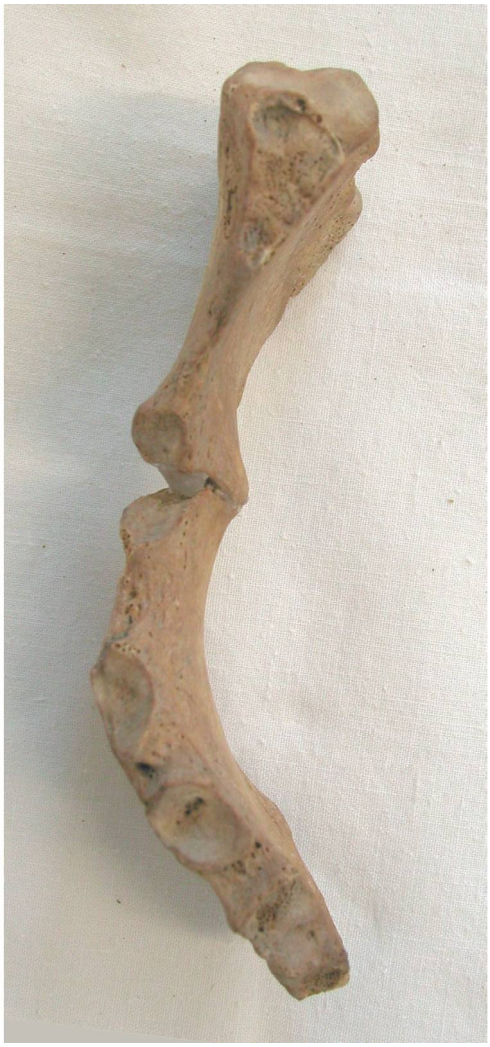

Figure 4. Grave 1414: Maturus female, pectus gallinaceum.

Among the 2272 adult skeletons, only 58 (2.55%) showed any signs of anaemia. In the latter group, the prevalence was higher in women (4.18%) than men (1.93%).

The occurrence of such alterations suggests iron-deficiency anaemia caused by malnutrition.

Developmental anomalies and diseases of the spine

Physiological curvature and poor body posture was only observed in ten cases, which may be explained by the fact that some of the skeletons are incomplete and fragmentary.

We have already mentioned some traces of developmental anomalies of the spine above. Different forms of sacralization could be detected in 26 cases, affecting primarily males. Five cases of lumbalization were found. Spina bihda is quite frequent (161 cases), affecting four times as many males as females. Considering all 872 pathological cases, the prevalence

Figure 5. Grave 993/a: Senium male, ankylosis of the right knee joint.

of spinal developmental anomalies is 26.61%. Spondyolisis is uncommonly frequent (24 cases), affecting males in more than 50% of the cases, and the majority of which (22) being of lumbar localisation.

Among the 160 diseased spines we found signs of inflammation in 17 (10.63%) cases. In comparison to the recent dataset, the prevalence of inflammation is much higher in Bâtmonostor.

82 (51.25%) cases of degenerative alterations were detected among the above mentioned 160 skeletons. Considering all 872 pathological findings, the total prevalence is 9.40%. The most common disorder is spondylarthrosis, which affected the cervical and thoracic vertebrae in 7-7 cases and the lumbar region in 47 cases, males and females in equal distribution. As there were twice as many male than female skeletons unearthed, spondylarthrosis seems to have been more frequent in women. Formation of osteophytes at the margin of the corpus vertebrae was also observed.

Also belonging to the degenerative group of disorders, Forestier-Rotes disease (the etiology of which being still unclear) was observed in 8 cases.

Block vertebrae were observed in the cervical region (5 cases), in the thoracic region (2 cases) and in the lumbar region (4 cases). In seven cases the fusion of two, in three cases the fusion of three vertebrae was observed. Triple fusions of vertebrae only occurred in the cervical region. In a special case lumbar and the lowest thoracic vertebrae were fused.

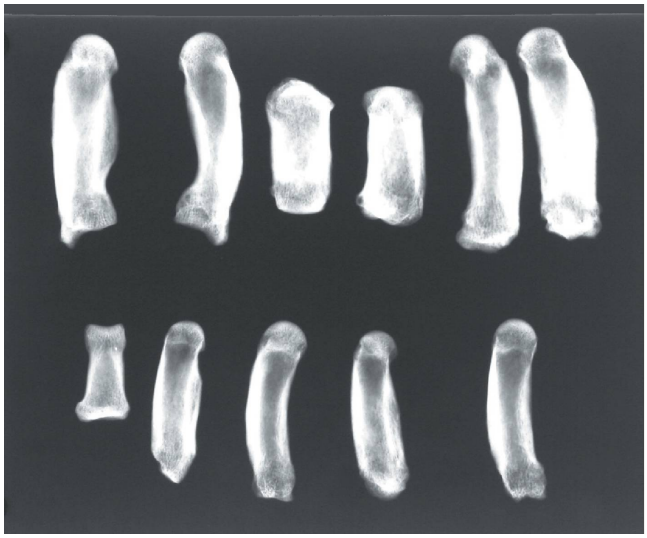

Figure 6. Grave 2528: Adultus male, Bamberger-Marie syndrome. X-ray picture of the metacarpals.

Herniated disk in the lumbar region was observed in three cases, Schmorl's hernia of the thoracic region in four, and of the lumbar region in nine cases.

Osteoporosis affected the vertebrae of an elderly woman. No tumours or traumatic lesions could be detected on any spines.

The findings in grave no. 2528 are especially worth mentioning. Large tumescences can be observed on the bones of the upper and lower arm, one phalanx and leg, which based on microscopic and X-ray scans show symptoms of Bamberger-Marie syndrome (hypertrophic osteoarthropathy) (Fig. 6). Such a case has never been described in excavated material in Hungary.

Bone tumors

We detected 24 primary and 1 metastatic bone tumor on the skeletal remains. Most of the tumors are benign, 19 cases of osteochondroma on 17 skeletons and a further three cases of osteoma were observed. There are two children, ten males, two females and three skeletons of undetermined sex among the 17 cases.

Osteosarcoma was diagnosed in two cases (Fig. 7). Metastatic bone tumour was observed on the ala ossis ilii, acetabulum and femora. The occurrence of benign bone tumors is quite rare in excavated materials, therefore, the cases mentioned above represent high prevalence.

We also found two cases of osteoma, one on the squamous part of the os temporale, the other at the tuber frontale. Both cases affected males. 15th-17th century sources refer to such bony growths as "human horns".

Figure 7. 16th section, stray find: osteosarcoma on a distal left femur.

Prevalence of enthesopathia

As the result of backbreaking labour of adults, we observed hyperplasia especially on the calcaneus, the patella, but also on all other limb bones. This hyperostosis can be considered as the natural adjustment of the human body.

According to our data, the total number of enthesopathic cases is 267, the calcaneus being affected in 238 cases, 213 of which were males. This prevalence - despite the fact that twice as many male skeletons were unearthed as female - clearly indicates that hyperostosis is characteristic to male skeletons.

This also supports the concept that the Bátmonostor population, and especially men, suffered from overburden of the muscles and joints, which is the indicative of hard physical work.

Diseases of the teeth, maxilla and mandíbula

Detailed examinations of the teeth or the oral cavity were not carried out. However, we noted some common anomalies during the examinations, namely: granulomas, massive plaque

formation, cysts, large-scale caries. In some cases, the traces of the arthritis of the caput mandibulae was observed. The prevalence of torus palatinus has already been referred to in the "Anatomical variations" section.

The biological similarity of the Bátmonostor findings to other series according to Penrose's method

The arithmetic average of ten measurements of the material from the Bátmonostor excavation was compared to 28 Hungarian series from the Arpadian- and Middle ages employing the Penrose-method. The material shows closest relation to the Ópusztaszer findings. Both series were unearthed from a graveyard around a monastery, but there is great chronological gap between the two. The findings at the Bátmonostor site and other sites show significant distance.

Taxonomic distribution

The prevalence of cromagnonids is 37.74% among males and 30.65% among females. Brachycephalous types occurred in 29.71% of the male and 33.86% of the female population. The relative prevalence of nordoids is quite low (19.81% of males, 18.55% of females). The rare presence of mediterranien races is suprising (12.74% of males, 16.94% of females).

Basically there is no difference between the taxonomic distribution of the sexes. The prevalence of alpine (12.26 of males, 12.90% of females) and pamirian types (13.21% of males, 16.13% of females) is remarkable.

As for the sexual distribution of the taxa, the population seems to be homogenous, while there prevalence of certain taxa greatly varies. In comparison to three sites from the Arpadian age (Szatymaz-Vasútállomás, Orosháza-Rákóczi telep, Békés-Povádzug) and Ópusztaszer, there are several similarities and differences as well.

Humanecoiogicai pesrpectives

Considering the analysis of human remains detailed above, we may well come to the conclusion that the findings derive from rather closed community of farmers. This concept is also supported by the taxonomic analysis, as the findings are fairly similar and we could only distinguish few different taxa. The population most probably lived under poor conditions, which is indicated by the high prevalence of severe diseases rarely found in historical anthropological samples. The large number of children refers to high infant mortality rate, which, however, is not surprising in the medieval ages. The most probable cause of death that left traces on the bones too was anaemia caused by malnutrition.

Discussion and Conclusion

The anthropological analysis of the findings of Bátmonostor raised several problems. As multiple burials were quite frequent, the excavation itself and the isolation of individuals became problematic. As a result of this, the exact number of persons buried cannot be determined. There were also quite a large number of incomplete skeletons, therefore the possibility of determining sex, age at death and taking measurements was rather limited. Nevertheless, we suppose that initially more individuals were buried here than the 3783 identified by us. This concept is also supported by the opinion of the site's archaeologist according to whom only approximately 80 % of the cemetery was opened up. However, the available skeletal material indicates that the graveyard belonged to a densely populated settlement, used for over 150 years. This cemetery could be considered as the largest medieval graveyard excavated in Hungary so far, so the series could serve as a very good comparative basis for studies on the anthropology of the medieval Hungarian populations.

Most certainly, there are further research possibilities considering this skeletal series; for instance, due to the enormous number of individuals our means were too limited to carry out investigations on dentition or growth rate of children. Therefore, all initiatives to perform new types of analyses and cooperation are welcome.

References

Acsádi Gy, Nemeskéri J (1970) History of human life span and mortality. Budapest

Biczó P (1978-1986) Bátmonostor-Pusztafalu (Bács-Kiskun m.). Régészeti Füzetek 31: 93-94., 32: 108-109., 33: 90., 34: 86., 35: 100., 36: 90., 37: 101., 38: 89., 39:79.

Farkas LGy (1972) Antropológiai praktikum 1. Paleoantropológiái metodikák. Szeged.

Józsa L, Farkas LGy, Rékó Gy (2004) A csontsérülések és szövődményeik gyakorisága a XIV-XV. Századokban. Magyar Traumatológia, Ortopédia, Kézsebészet, Plasztikai Sebészet 47:141-147.

Kőhegyi M (1967) Bátmonostor-Pusztafalu (Bács-Kiskun m., Bajai járás). Régészeti füzetek 20:71-72.

Nemeskéri J, Harsányi L, Acsádi Gy (I960) Methoden zur Diagnose des Lebensalter von Skelettfunden. Anthrop Anz 24:71-88.

Penrose LS (1954) Distance, size and shape. Ann Eugen 18:337-343.

Sjövold T (1990) Estimation of stature from long bone utilizing the line of organic correlation. Human Evol 5:431-447.

Stloukal M, Hanáková H (1978) Die Länge der Längsknochen altslawischer Bevölkerungen unter besonder Berücksichtigung von Wachstumsfragen. Homo 29:53-69.

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ARTICLE

Responses of antioxidant defense capacity and photosynthesis of bean *(Phaseolus vulgaris* L.) plants to copper and manganese toxicity under different light intensities

Roghieh Hajiboland*, Behrokh D Hasani

Plant Science Department, University of Tabriz, Tabriz, Iran

ABSTRACT Effects of toxic concentrations of Cu and Mn were studied in bean *(Phaseolus vulgaris)* **plants under different light intensities. Exposure of plants to 100 pM Cu, inhibited their growth up to 79%, while similar concentrations of Mn caused only a slight (21%) reduction of plants dry weight. Net assimilation rate (A) was inhibited with similar extent by both heavy metals and F/F_0 ratio decreased particularly by Mn treatment. With the exception of ascorbate peroxidase in Cu treated plants, activity of catalase, superoxide dismutase and glutathione reductase were stimulated by both heavy metals with similar extent. Change in guaiacol peroxidase activity was correlated with growth response, increased by Cu but was not affected by Mn toxicity. Similar to peroxidase activity, accumulation of proline monitored the stress conditions but did not cause more protection against Cu toxicity. On the other hand, concentration of H_20_2 and malondialdehyde indicated a higher oxidative stress simultaneous with higher growth in the Mn than Cu treated plants. Results imply that, growth response to Cu and Mn could be reflected neither by the chlorophyll fluorescence nor the net assimilation rate. Additionally, antioxidant defense capacity did not involve in different response to Cu and Mn in bean plants.**
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KEY WORDS

antioxidant capacity
Cu toxicity
chlorophyll fluorescence
gas exchange
light Intensity
Mn toxicity

Copper and manganese are essential micronutrients for plants. However, excess amounts of these heavy metals induce a wide range of biochemical effects and physiological processes and alter photosynthesis, pigment synthesis, protein metabolism and membrane integrity (Hall 1994). Usual causes of high levels of Cu in the soil are mining activities or the prolonged application of Cu-based fungicides. In contrast, manganese (Mn) is not a common pollutant in soils, but various soil conditions often present in acid and volcanic soils or submergence can lead to Mn reduction and create Mn toxicity in many natural and agricultural systems (Foy et al. 1978).

Toxic trace pollutants can induce many alterations in plant cells (Woolhouse 1983), but it is difficult to draw a general mechanism about the physiology of stress, since metal toxicity results from complex interaction of metal ions with several metabolic pathways. However, one of the underlying causes of tissues injury following exposure of plants to Cu (Chen et al. 2000) and Mn (González et al. 1998) is the increased accumulation of reactive oxygen species mediated-oxidative stress (De Vos and Schat 1991).

Exposure to various photooxidative stress factors can stimulate the plant free radical scavenging systems. The activity of one or more antioxidative enzymes can increase and the concentration of low molecular weight antioxidants can be elevated in response to oxidative stress. Such changes are usually correlated with an improved tolerance (Gressel and Galun, 1994). Accordingly, pre-exposure to sub-lethal levels of one kind of oxidative stress may provide a better acclimation to other kinds of oxidative stress (cross-resistance) due to the activation of the protection system (Schöner and Krause 1990; Bridger et al. 1994).

The deleterious effects resulting from cellular oxidative state may be alleviated by detoxifying enzymes, such as superoxide dismutase (SOD), catalase (CAT), and enzymes of ascorbate-glutathione cycle, ascorbate peroxidase (APX) and glutathione reductase (GR). Peroxidases are considered to be heavy metal stress-related enzymes (Karataglis et al. 1991) and can be used as stress markers in metal poisoning situations (Mocquot et al. 1996; Chaoui et al. 2004). However, increase in their activity is thought to be a common response and can protect plants to various stress factors (Gaspar et al. 1985; Castillo 1992).

Proline accumulation accepted as an indicator of environmental stresses, is also considered to have important protective roles (Alia and Saradhi 1991). Accumulation of free proline in response to heavy metal exposure seems to be wide-spread among plants (Schat et al. 1997). The

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Corresponding author. E-mail: ehsan@tabrizu.ac.ir

functional significance of proline accumulation would lie in its contribution to water balance maintenance (Costa and Morel 1994) scavenging of hydroxyl radicals (Smirnoff and Cumbes 1989) and metal chelation in the cytoplasm (Farago and Mullen 1979).

One of the most important effects of reactive oxygen species is the loss of membrane integrity (Vichnevetskaia and Roy 1999). Thylakoid lipids are susceptible to oxidation due to predominance of polyunsaturated fatty acids (Gounaries et al. 1986) and the availability of oxygen producing free radicals around functional PSII (Kyle 1987). Therefore, photosynthetic membranes in chloroplasts are the most susceptible structures in plants grown under conditions of oxidative stresses. Reactive oxygen species not only are produced in response to heavy metal stress in shoot and root, but also higher light intensity alone or in combination with heavy metal toxicity could led to a severe oxidative damage of leaves in stressed plants.

It was reported that, response of plants to excess Mn is affected by light intensity (González et al. 1998). However, reports on the effect of high light intensity on Mn toxicity are contradictory, including increasing (Horiguchi 1988; Nable et al.1988) or lessening toxicity symptoms (Wissemeier and Horst 1992). A factor that complicates the interpretation of previous studies of the effect of light intensity on Mn-toxicity symptoms is the fact that plants grown in low light usually accumulate less foliar Mn than those grown at a higher light intensity (Me Cain and Markley 1989). In contrast to Mn, there is no report on the effect of growth under various light intensities on Cu tolerance of plants.

Copper is a potent inhibitor of photosynthesis and chlorophyll synthesis (Fernandes and Henriques 1991) in some plants such as barley and spinach. However, no significant effect of Cu toxicity on chlorophyll synthesis in maize was reported (Chaoui et al. 1997). Similarly, reports on the effect of Mn toxicity on chlorophyll content of plants, is contradictory, from no change (Wissemeier and Horst 1992) to severe chlorosis (Horiguchi 1988; Nable et al.1988) depending on plant species.

Copper has been shown to increase susceptibility to photo-inhibition particularly in intact leaves (Pätsikkä et al. 1998) but the underlying mechanism has remained unclear. It has been known that high concentrations of copper when added to the incubation medium of isolated thylakoids, inhibit PSII electron transfer activity on the acceptor side (Yruela et al. 1996) and finally cause the release of the external polypeptides of the oxygen-evolving complex on the donor side of PSII (Pätsikkä et al. 2001). Some authors suggested that excess copper in the growth medium did not cause loss of photoprotection, but reduced chlorophyll content causes the high photosensitivity of PSII in copper treated plants (Pätsikkä et al. 2002). Very limited data are available concerning photochemistry of leaves under Mn toxicity.

Changes in chlorophyll fluorescence emissions are indications of changes in photosynthetic activity (Kautsky et al. I960). Chlorophyll fluorescence gives information about the state of Photosystem II and about the extent to which PSII is using the energy absorbed by chlorophyll and the extent to which it is being damaged by excess light (Maxwell and Johnson 2000). The flow of electrons through PSII is indicative of the overall rate of photosynthesis and is an estimation of photosynthetic performance. To our knowledge, this is the first study of the potential impact of Cu and Mn toxicity in combination with high light intensity on photosynthesis.

There are only limited data available concerning response of photosynthesis capacity of plants to heavy metal toxicity. It is still an open question as to whether stomatal closure is the main factor inhibiting photosynthesis and biomass production under heavy metal stress. It is also plausible that heavy metals toxicity affects the photochemistry of leaves and inhibits biophysical processes of photosynthesis.

Because of highly different physicochemical properties of Mn and Cu, as well as different behavior regarding chelation by organic molecules and binding to the cell wall (Marschner 1995), it is necessary to under taken a comparative study of Mn and Cu toxicity on biochemical processes of plants particularly antioxidant defense system and photosynthesis processes. In this work we studied the response of bean plants to Cu and Mn toxicity as influenced by dual effect of metal toxicity and high light intensity. The main objective of this work was the evaluation of the importance of inducible or constitutive antioxidant defense capacity of plants and various physiological traits of photosynthesis in growth and biomass production of bean plant under heavy metal toxicity.

Materials and Methods

Seeds of one cultivar of bean *(Phaseolus vulgaris* cv. Naz) was used in this study were provided by Seed and Plant Improvement Institute (SPII) (Karaj, Iran).

Plant cultures and treatments

The experiments were conducted in a growth chamber with a temperature regime of 25/18°C day/night, 14/10 h light/dark period and relative humidity of 70/80%. Surface-sterilized seeds were germinated in the dark on sand, moistened with distilled water and $CaS0_4$ at 0.05 mM. The 7-day-old seedlings with uniform size were transferred to hydroponic culture in plastic container with 2L of nutrient solution (50%) and pre-cultured for 3 days. Copper and Mn treatments were started for 10-days-old plants, consisted of three levels of $CuS0_4$or $MnS0_4$at 0 (control), 50 and 100 pM. Plants were treated for 14 days and nutrient solutions were completely changed every 3 days. Composition of the nutrient solutions were used according to Neumann et al. (1999), pH was 6.5 and adjusted every day.

For study of the effect of different light intensities, plants were grown simultaneously under three light intensities: low light (LL=100 pmol m⁻ V¹ PPFD), intermediate light (IL=500 pmol m⁻V¹ PPFD) and high light (HL=800 pmol m'V¹ PPFD) intensities supplied by fluorescent lamps. The incident photosynthetic photon flux density (PPFD) was measured by a quantum sensor attached to the leaf chamber of the gas exchange unit.

Harvest

After 14 days treatment, plants were harvested. For removing of the apoplasmic Cu and Mn from roots, plants were placed for 20 min in 5 mM CaC^+25% nutrient solution. Thereafter, plants were divided into shoots and roots, roots were washed with distilled water, weighed and blotted dry on filter paper and dried at 70°C for 2 days to determine plant dry weight.

For the determination of Cu and Mn content, oven-dried samples were ashed in a muffle furnace at 550°C for 8h, thereafter samples were resuspended in 2 ml 10% HC1 and made up to volume by double-distilled water. Copper and Mn concentration was determined by atomic absorption spectrophotometry (Shimadzu, AA 6500).

Chlorophyll concentration was measured spectrophotometrically in the third youngest leaf after weighing and a 48 h extraction in N,N-dimethylformamide. The absorbance of chlorophyll was measured at 644, 647 and 603 nm using spectrophotometer (Specord 200, Analytical Jena, Germany) and chlorophyll concentration was calculated using following formula (Moran 1982). Thereafter, values were recalculated on the fresh weight basis of leaves.

Chlorophyll a (pg ml¹) = 12.91 A_{644} - 2.12 A_{647} - 3.85 A_{603}

Chlorophyll b (pg ml¹) = -4.67 A_{644} + 26.09 A_{647} - 12.79 A_{603}

Another group of plants were used for measurement of gas exchange parameters and chlorophyll fluorescence.

Determination of gas exchange parameters

$C0_2$ assimilation and transpiration rates of attached leaves were measured with a calibrated portable gas exchange system (LCA-4, ADC Bioscientific Ltd., UK) always between 9:00 A.M. and 15:00 P.M. Measurements were carried out on one mature, fully expanded and attached leaf from *4* plants per treatment illuminated with the treatment specific light intensity (LL, IL or HL). With the exception of PPFD, no microenvironmental variable inside the chamber was controlled.

The net photosynthesis rate by unit of leaf area (A) and the stomatal conductance to water vapor *(g)* were calculated using the values of $C0_2$ and humidity variation inside the chamber, both measured by the infrared gas analyzer of the portable photosynthesis system. Other parameters calculated were the ratio of intercellular air space and atmospheric $C0_2$ molar fraction *(Ci/Ca)* and photosynthetic water use efficiency *(WUE)* was determined by the ratio of net photosynthesis rate (A) to transpiration rate *(E) (WUE=A/E)*.

Determination of chlorophyll fluorescence

Chlorophyll fluorescence parameters were recorded in parallel for gas exchange measurements in the same leaf, using a portable fluorometer (FIM, ADC Bioscientific Ltd., UK). Leaves were acclimated to dark for 30 min before measurements were taken. Initial (F_0), maximum (F_m), variable ($F_v = F_m - F_0$) as well as F_v/F_m and F_v/F_0, ratios were recorded.

Experiments were under taken in complete randomized block design with *4* replications. Statistical analyses were carried out using Sigma stat (3.02) with Tukey test ($p<0.05$).

Enzyme assays

Fresh leaf samples were used for enzyme extraction and measurement of protein and metabolites. Whole shoot was ground in extraction buffer using pre-chilled mortar and pestle. Each enzyme assay was tested for linearity between the volume of crude extract and the measured activity. Change in the absorbance of substrates or products (depending on assay protocol) was measured using spectrophotometer (Specord 200, Analytical Jena, Germany). Activity of enzymes were calculated on the protein concentration basis (specific activity).

Ascorbate peroxidase: The enzyme was extracted in 50 mM phosphate buffer (pH=7.0). The activity of ascorbate peroxidase (APX, EC 1.11.1.11) was measured using modified method of Boominathan and Doran (2002). The reaction mixture consisted of 50 mM sodium phosphate buffer (pH=7.0) containing 0.2 mM EDTA, 0.5 mM ascorbic acid (Sigma), 50 mg of BSA (Sigma), and crude enzyme extract. The reaction was started by addition of H_20_2 at final concentration of 0.1 mM. Oxidation of ascorbic acid as a decrease in absorbance at 290 nm was followed *2* min after starting the reaction. The enzyme activity was calculated using an absorbance coefficient for ascorbic acid of 2.8 mM⁻'em¹. One unit of APX oxidizes ascorbic acid at a rate of 1 pmol min¹ at 25°C.

Catalase: Catalase (CAT, EC 1.11.1.6) activity was assayed spectrophotometrically by monitoring the decrease in absorbance of H_20_2 at 240 nm (Simon et al. 1974). The enzyme was extracted in 50 mM phosphate buffer (pH=7.0). The assay solution contained 50 mM phosphate buffer and 10 mM H_20_2. The reaction was started by addition of enzyme aliquot to the reaction mixture and the change in absorbance was followed *2* min after starting the reaction. Unit activity was taken as the amount of enzyme, which decomposes 1 mol of H_20_2 in one min.

Peroxidase: Peroxidase (POD, EC 1.11.1.7) activity was determined using the guaiacol test (Chance and Maehly 1955). The tetraguaiacol formed in the reaction has an absorption maximum at 470 mm, and thus the reaction can be readily followed photometrically. The enzyme was extracted

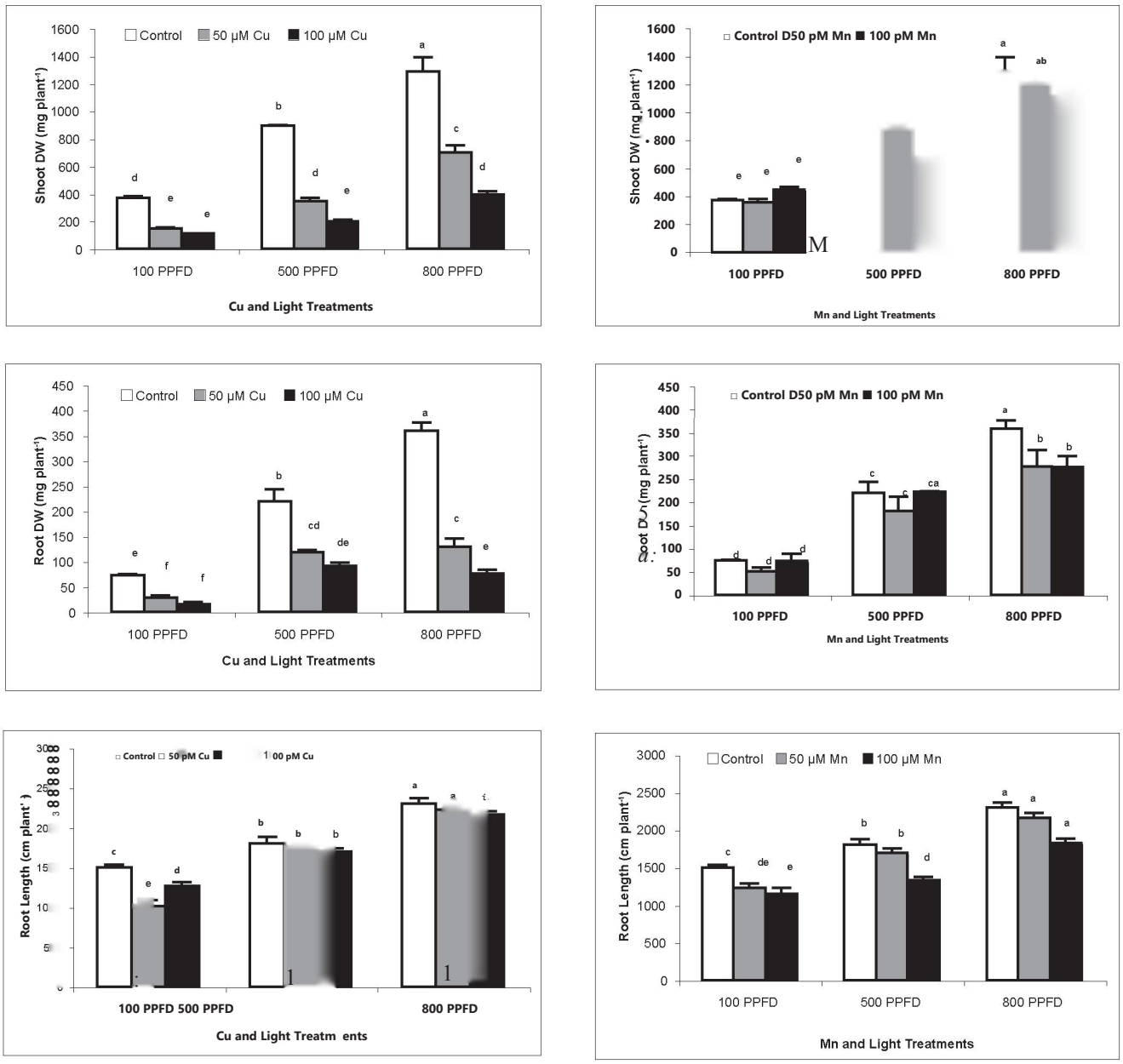

Figure 1. Effect of Cu and Mn treatments on shoot and root dry weight and root length of bean *(Phaseolus vulgaris* L.) plants under three light conditions. Each value is the mean of 4 repetitions ± SD. Bars denoted with the same letter are not significantly different (P<0.05).

by 10 mM phosphate buffer (pH=7.0), and assayed in a solution contained 10 mM phosphate buffer, 5 mM H_20_2 and 4 mM guaiacol. The reaction was started by addition of the enzyme extract at 25°C and was followed 2 min after starting the reaction. The enzyme unit was calculated as enzyme protein required for the formation of 1 pmol tetraguaiacol for 1 min.

Superoxide dismutase: Total superoxide dismutase (SOD, EC 1.15.1.1) activity was determined according to Giannopolitis and Ries (1977). The enzyme was extracted in 25 mM HEPES (pH=7.8) and 0.1 mM EDTA, the homogenate was centrifuged at15 000 *g* for 15 min. Test tubes containing 25 pi of enzyme extract, 25 pi extraction buffer and 450 pi of the reaction mixture were incubated in a growth chamber at 22°C and at a light intensity of 400 pmol $m_{-2}s_{-1}$. The reaction buffer contained 25 mM HEPES (pH=7.6), 0.1 mM EDTA, 50 mM Na_2C0_3 (pH=10.2), 12 mM L-methionine, 75 pM NBT and 1 pM riboflavin. The reaction was started by removing a dark plastic foil from the surface of samples and continued for 10 min. One unit of SOD was defined as the amount of

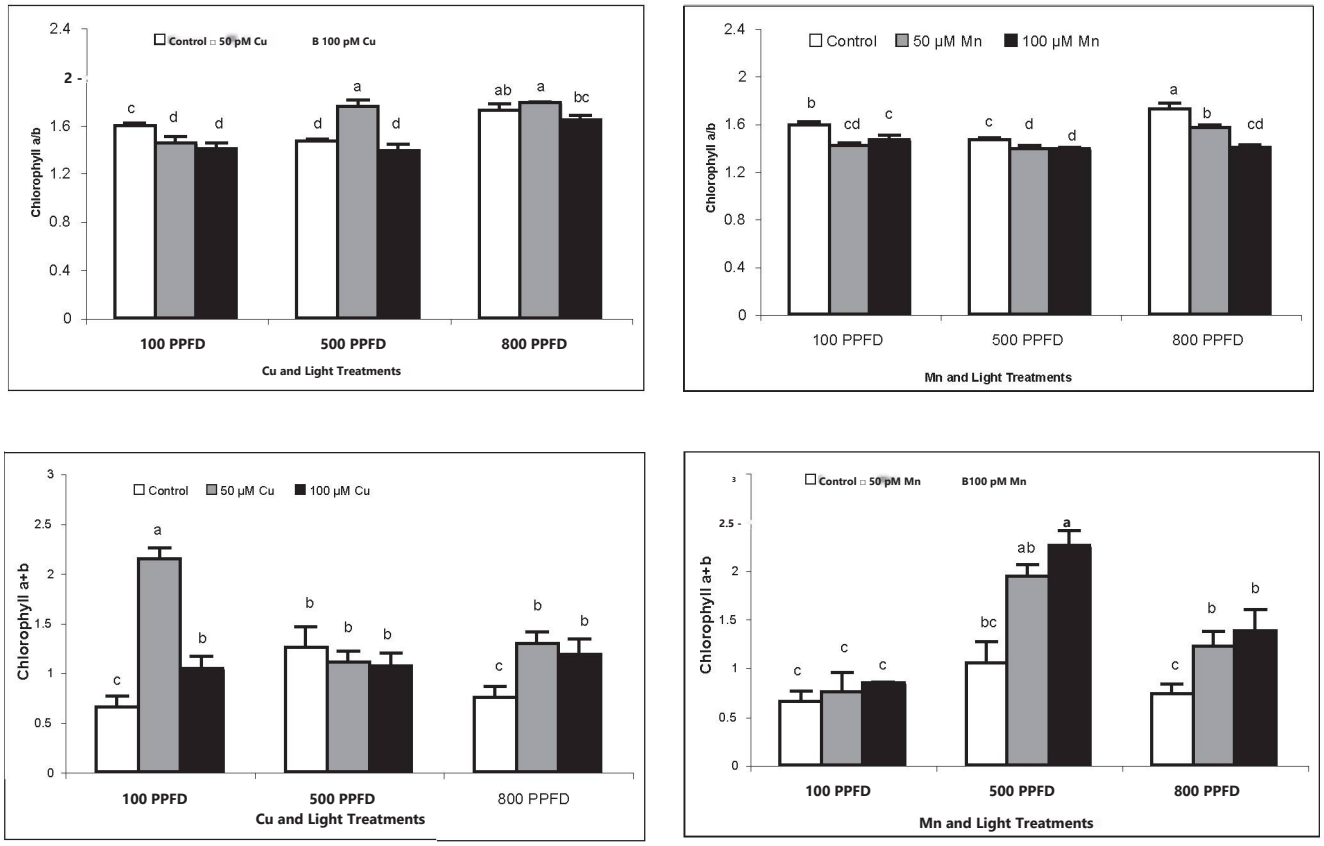

Figure 2. Changes in the leaf chlorophyll a/b ratio and chlorophyll a+b amounts in bean *(Phaseolus vulgaris* L.) plants treated by Cu or Mn and grown under three light conditions. Each value is the mean of 4 repetitions ± SD. Bars denoted with the same letter are not significantly different (P<0.05).

enzyme required to induce a 50% inhibition of NBT reduction as measured at 560 nm, compared with control samples without enzyme aliquot.

Glutathione reductase: The enzyme was extracted in 50 mM phosphate buffer (pH=7.0) containing 5 mM EDTA and 2% (w/v) of insoluble Polyvinylpolypyrrolidone (PVPP). The extract was centrifuged at 15 000 *g* in 4°C for 20 min. The activity of glutathione reductase (GR, EC 1.6.4.2) was assayed by following the oxidation of NADPH at 340 nm (extinction coefficient 6.2 mM cm^{1}) as described by Foyer and Halliwell (1976). The reaction mixture contained 100 mM Tris-HCl (pH=7.8), 2.0 mM EDTA, 0.05 mM NADPH, 0.5 mM oxidized glutathione (GSSG) and 50 pi of enzyme extract at 25°C. One unit of enzyme activity was calculated as enzyme protein required for oxidation of one pmol NADPH in 1 min.

Other assays

Total protein concentration: Soluble proteins were determined as described by Bradford (1976) using a commercial reagent (Sigma) and BSA (Merck) as standard.

Total amino acids: Content of total free a-amino acids was assayed using ninhydrin colorimetric method (Hwang and Ederer 1975). Leaf tissues were homogenized using ice cold 50 mM phosphate buffer (pH=6.8). The homogenate was centrifuged at 18 000 *g* for 20 min. Ninhydrin reagent (1:5 diluted solution of 350 mg in 100 ml ethanol) was added to sample solution and after gentle stirring was incubated for 4-7 min at 80-100°C in a water bath. After cooling to room temperature in a water bath, the absorbance was recorded at 570 nm. Glycine was used for production of standard curve.

Proline concentration: Proline was extracted and its concentration determined by the method of Bates et al. (1973). Leaf tissues were homogenized with 3% sulfosalicylic acid and the homogenate was centrifuged at 3000 *g* for 20 min. The supernatant was treated with acetic acid and acid ninhydrin, boiled for 1 h, and then absorbance at 520 nm was determined. Proline (Sigma) was used for production of standard curve.

Hydrogen peroxide: The concentration of H_20_2 was determined using methods described by Patterson et al. (1984). 1-1.5 g of leaf was homogenized with 0.2 g activated charcoal

Table 1. Effect of Cu and Mn treatment on the shoot concentration of Cu and Mn (|jg g-1 DW) in bean *(Phaseolus vulgaris* L. cv. Naz) plants grown under three light conditions including low (LL), intermediate (IL) or high (HL) for 14 days in hydroponic medium. The means refer to 4 repetitions ± SD. Data in each column followed by the same letter are not significantly different (P<0.05).

Light Treatment	Cu/Mn (JM)	Cu Concentration	Mn Concentration
LL	0	794±34<	120±25 d
	100	7239±52 d	854±12 d
IL	0	1100±43e	152±12 d
	100	8456±82 »	1017±26»
HL	0	1289±63d	239±33d
	100	12300±125 ■»	1321±45 d

(Sigma) and 5 ml of 5% w/v trichloroacetic acid (TCA) in an ice bath using a prechilled mortar and pestle. The homogenates was hltered through four layers of cheesecloth and centrifuged at 14 000 *g* for 15 min at 4°C. The supernatant was then hltered through a 0.45 pM hlter (Millipore). The colorimetric reagent was a 1:1 v/v mixture of 0.6 mM 4-(2-pyridylazo) resorcinol (disodium salt) and 0.6 mM potassium titanium-oxalate. To a known volume of supernatant, 1 ml of colorimetric reagent was added and the mixture was incubated at 45°C on a heating plate for 60 min. The absorbance was measured at 508 nm against a reference solution containing 50 pi of 50% w/v TCA and 1.95 ml of 100 mM potassium phosphate buffer (pH=8.4). The concentration of H_20_2 was determined from a standard curve.

Malondialdehyde assay: Lipid peroxidation was estimated from the amount of malondialdehyde (MDA) formed in a reaction mixture (Heath and Packer 1968). Leaf tissues were homogenized (1:5) in 0.1% w/v TCA. The homogenate was centrifuged at 10 000 *g* for 5 min. To 1 ml of the supernatant, *4* ml of 20% w/v TCA containing 0.5% w/v thiobarbituric acid (Sigma) was added. The solution was heated at 95°C for 30 min and then quickly cooled on ice. The mixture was centrifuged 10 000 *g* for 15 min and the absorbance measured at 532 nm. MDA levels were calculated from a 1,1,3,3-tetraethoxypropane (Sigma) standard curve (Boominathan and Doran 2002).

All experiments were conducted using *4* independent replications. Statistical analyses were carried out using Sigma stat (3.02).

Results

Dry matter production and heavy metal accumulation

In general, plants grown under higher light intensity have higher dry matter of shoot and root and root length. Toxic concentrations of Cu decreased shoot and root growth drastically, this reduction was 71% and 69% for shoot, 79% and 79% for roots dry weight of LL and HL plants respectively. Root length was affected much less than shoot and root dry weight and was only reduced 16% and 6% for LL and HL plants respectively. Tolerance of plants to toxic concentrations of Mn, was much higher compared to Cu. Shoot dry weight was reduced by 100 pM Mn only 21% and 14% in LL and HL plants respectively. Root dry weight was not affected in LL plants treated by Mn concentration as high as 100 pM, and in HL plants rather an increase up to 23% was observed. As it is obvious from growth data, light intensity did not influence Cu and Mn tolerance of bean plants, so that the extent of inhibitory effect of toxic concentrations of Cu and Mn in the medium was similar between LL, IL and HL plants (Fig. 1). Growing under higher light intensity increased accumulation of both Cu and Mn in shoots. Copper accumulated in shoots up to 12 mg g^{l} DW, the corresponding amount for Mn was only 1.3 mg g^{l-} DW (Table 1).

Chlorophyll concentration

In the absence of Cu and Mn, chlorophyll (a+b) concentration increased under light intensity of 500 PPFD (IL) but decreased with further increasing of light intensity. Chlorophyll (a+b) concentration was influenced by both Cu and Mn treatment. Moderately toxic levels of Cu (50 pM) increased chlorophyll (a+b) concentration in LL plants, resulting from higher reduction of leaf growth than chlorophyll concentration. Cu treatment at 50 pM, caused an increase of the ratio of chlorophyll a/b in IL and HL plants, which was the result of increased values of chlorophyll a in Cu treated leaves (data of individual values of chlorophyll a and b were not shown). Manganese treatment caused a significant increase of chlorophyll a+b concentration in plants grown under higher light conditions. In contrast to Cu, Mn caused a reduction of the ratio of chlorophyll a/b, which was the result of increasing values of chlorophyll b values of Mn treated leaves (Fig. 2).

Gas exchange parameters

As expected, net assimilation rate (A) was higher in plants grown under higher light intensity. Cu and Mn treatment of plants affected negatively the net assimilation rate, the reduction were 40% and 71% in response to Cu treatment, 50% and 76% in response to Mn treatment for LL and HL plants respectively. Therefore, plants were much sensitive to inhibitory effect of heavy metals treatments when grown under higher compared to lower light intensities. Water loss via transpiration was not affected by Cu and Mn treatment significantly, however, increase in transpiration due to growth under higher light intensities was lower in heavy metal treated plants. Transpiration was 3.5 times higher in HL compared to LL in control plants, while the corresponding value for Cu treated plants was only 2.5 and for Mn treated ones was 2.0.

Table 2. Gas exchange parameters including net photosynthetic rate *(A),* transpiration (£), the ratio of intercellular air space and atmospheric $C0_2$ molar fractions (C/'/Ca), stomatal conductance to water vapor (gs) and instantaneous water use efficiency *(WUE)* and chlorophyll fluorescence parameters including F_V/F_m and F/F_0 of bean *(Phaseolus vulgaris* L.) treated with CuorMn under three light conditions. The means refer to 4 repetitions ± SD. Data in each column followed by the same letter are not significantly different (P<0.05).

	Cu/Mn (MM)	Cu Treatment			Mn Treatment		
		LL	IL	HL	LL	IL	HL
A (pmol m^2 $s^{'1}$)	0	20±9 ^{a}	23±14^a	42±25^a	20±9^a	23±14^a	42±25^a
	50	10±3 ^{a}	12±7 ^{a}	10±4^b	12±4^a	10±5 ^{a}	22±9 ^{ab}
	100	12±6 ^{a}	12±5 ^{a}	12±4^b	10±7 ^{a}	7±5 ^{a}	10±4^b
E (mmol m^2 s^1)	0	0.35±0.12 ^{a}	0.75±0.21 ^{a}	1.22±0.71 ^{a}	0.35±0.12 ^{a}	0.75±0.21 ^{a}	1.22±0.71 ^{a}
	50	0.35±0.01 ^{a}	0.73±0.51 ^{a}	0.93±0.51 ^{a}	0.38±0.11 ^{a}	0.74±0.20 ^{a}	0.95±0.50^a
	100	0.37±0.11 ^{a}	0.63±0.30 ^{a}	0.94±0.41 ^{a}	0.41±0.20^a	0.71±0. 01 ^{a}	0.83±0.30^a
Ci/Ca	0	1.01±0.45 ^{a}	0.89±0.31 ^{a}	0.95±0.50^a	1.01±0.45 ^{a}	0.89±0.31 ^{a}	0.95±0.50^a
	50	0.85±0.38 ^{a}	1.16±0.63 ^{a}	1.25±0.48^a	0.92±0.32 ^{a}	0.68±0.21 ^{a}	0.98±0.32 ^{a}
	100	0.85±0.32 ^{a}	1.10±0.88 ^{a}	0.97±0.34^a	0.86±0.42 ^{a}	0.63±0.33 ^{a}	1.08±0.45 ^{a}
g_s(mol m^{-2} s^1)	0	19±10^a	24±13^a	41±24^a	19±10^a	24±13^a	41±24^a
	50	11±5 ^{a}	16±10^a	32±14^a	16±8^a	20±13^a	31±12^a
	100	12±8 ^{a}	14±7 ^{a}	35±21^a	14±9^a	19±7 ^{a}	33±18^a
WUE (pmol mol'^{1})	0	57±8 ^{a}	31±5 ^{a}	35±8^a	57±8^a	31±5 ^{a}	35±8^a
	50	29±7 »	16±6»	11±5 »	32±8^b	14±4»	23±4^b
	100	32±6 »	19±5 ^{ab}	13±6^b	24±9^b	10±4^b	12±5 ^{b}
Fv/Fm	0	0.69±0.01 ^{a}	0.72±0.04 ^{a}	0.82±0.05 ^{a}	0.70±0.04^a	0.73±0.05 ^{a}	0.83±0.04^a
	50	0.66±0.02 ^{a}	0.70±0.03 ^{a}	0.80±0.02 ^{a}	0.67±0.04^a	0.71±0.05 ^{a}	0.81±0.05 ^{a}
	100	0.65±0.01 ^{a}	0.70±0.04 ^{a}	0.79±0.03 ^{a}	0.64±0.03 ^{a}	0.69±0.04 ^{a}	0.78±0.04^a
F_V/F_0	0	2.21±0.08 ^{a}	2.60±0.05 ^{a}	4.56±0.18^a	2.21±0.08^a	2.60±0.06 ^{a}	4.56±0.08^a
	50	1.96±0.04»	2.37±0.14^b	4.33±0.11 ^{a}	1.89±0.09»	2.25±0.11 ^{b}	4.13±0.13 ^{b}
	100	1.88±0.04»	2.33±0.08 ^{b}	3.70±0.12 ^{b}	1.63±0.09 ^{c}	2.13±0.13 ^{b}	3.27±0.15 ^{c}

Stomatal conductance of leaves was only influenced by light intensity and a gradual reduction of g_s values in response to Cu and Mn treatment was mainly in tendency and not significant. Water use efficiency was lowered by higher light intensity, Cu and Mn treatment exerted also similar effect, but the changes were mainly in tendency and not significant (Table 2).

Chlorophyll fluorescence

In response to treatment with both of Cu and Mn, F_g values increased which was observed under all three light conditions applied. Values of F_m were decreased in Cu and Mn treated plants, but only under the lowest light intensity applied in the experiment (PPFD=100 pmol $m^{-}V^1$). Under higher light intensities F_m values diminished in response to both of Cu and Mn treatment. Treatment by Cu and Mn caused reduction of F_v values, particularly under higher light conditions. Changes of F_g, F_m and F_v values were more pronounced in response to Cu than Mn treatment. T_m values were also decreased in heavy metal treated plants. The ratio of F_v/F_m remained mainly unchanged in response to Cu and Mn treatment. In contrast, the ratio of F_v/F_g decreased significantly in response to both Cu and Mn treatment. This reduction was 15% and 19% for Cu- and 26% and 28% for Mn-treated plants (Table 2).

Activity of antioxidant enzymes

Activity of APX responded to light intensity and both heavy metals. Higher light intensity caused a reduction in the activity of APX in control as well as Cu treated plants. Cu treatment induced reduction of APX activity in tendency or significant. In contrast, Mn treatment not only did reduce APX activity, but also resulted in a significant increase of the activity under IL and HL intensity. Catalase, SOD and GR activity responded with similar manner, in which both of increasing light intensity and the presence of heavy metals induced an increase in their activity. Similar extent of increase was observed in response to Cu and Mn. Similar with APX, activity of POD responded differently depending on heavy metals, a significant increase of POD activity was observed in response to Cu, while Mn treatment did not affect it. In contrast to APX, CAT, SOD and GR, light intensity did not influence activity of POD either in Cu or Mn treated plants (Fig. 3).

Concentration of oxidant and antioxidant metabolites

Only the highest light intensity induced a significant accumulation of proline in control plants. Copper treatment of plants caused a drastic rise of proline concentration, however, the

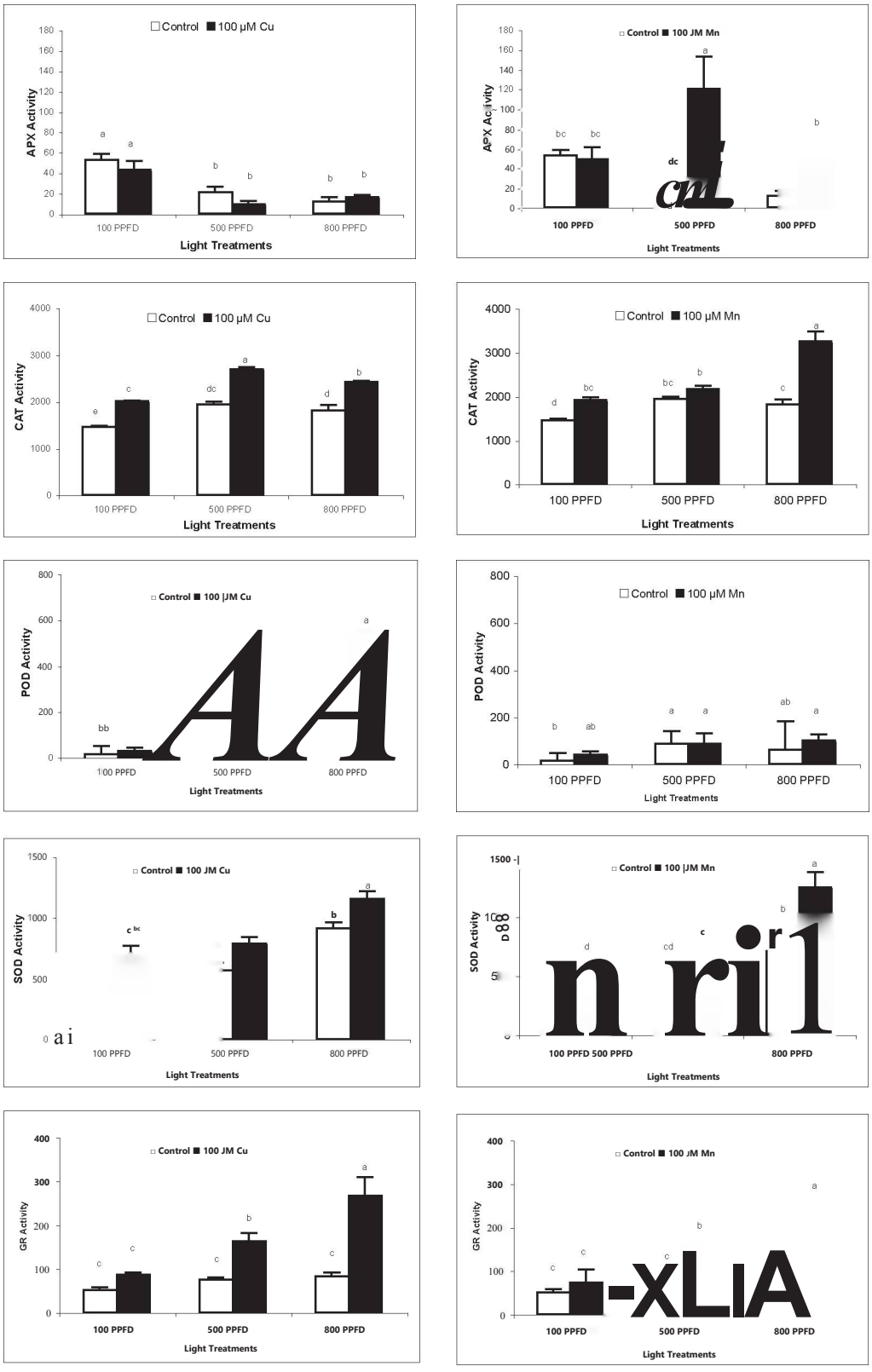

Figure 3. Effect of Cu and Mn treatment on the activity (Unit mg "[1] Pro. min'[1]) of ascorbate peroxidase (APX), catalase (CAT), guaiacol peroxidase (POD), superoxide dismutase (SOD) and glutathione reductase (GR) in leaves of bean *(Phaseolus vulgaris* L.) plants grown under three light conditions. Each value is the mean of 4 repetitions ± SD. Bars denoted with the same letter are not significantly different (P<0.05).

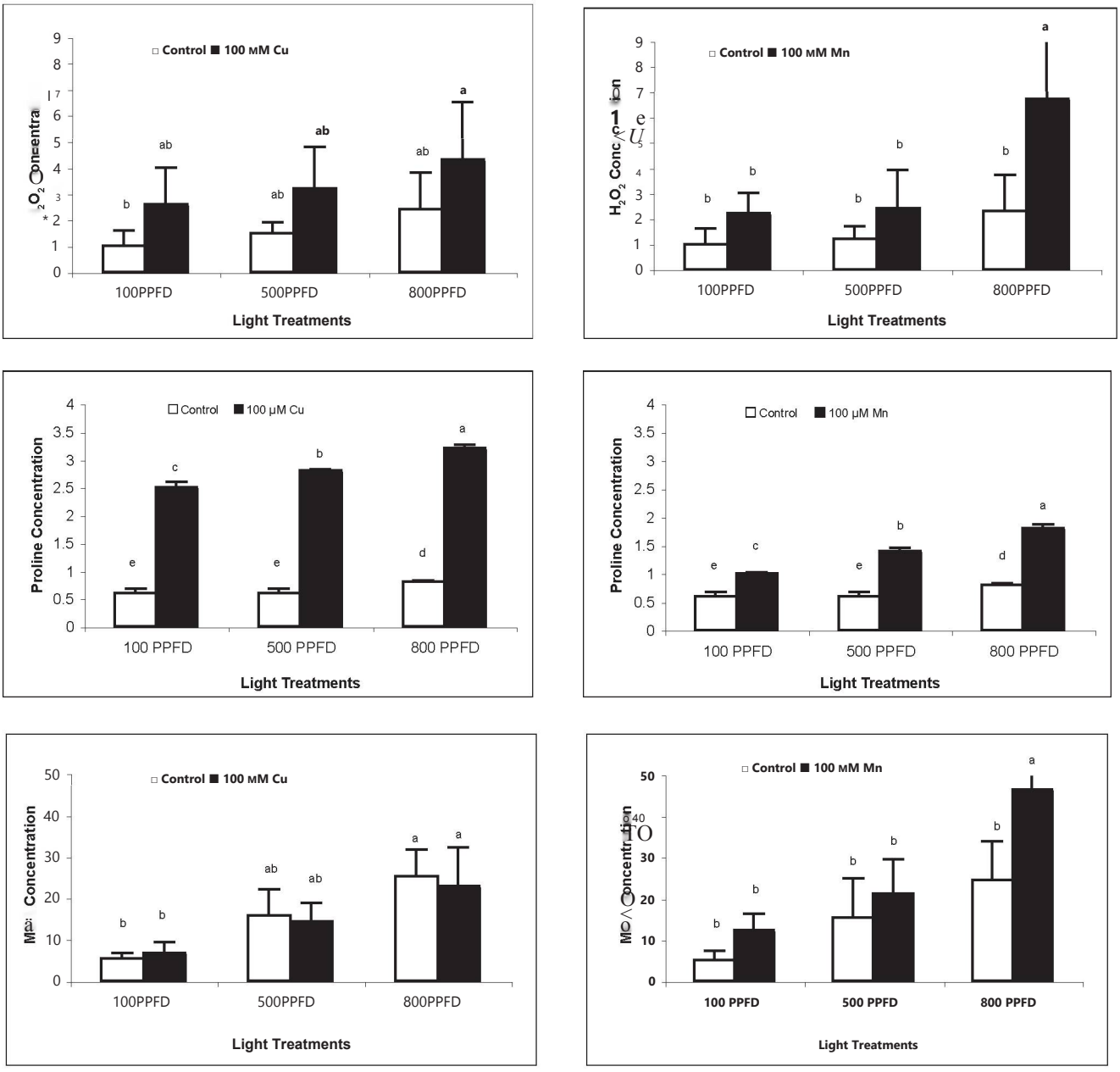

Figure 4. Effect of Cu and Mn treatment on the concentration of proline (pM g^{-1} FW), hydrogen peroxide (H_2O_2) (pM H_2O_2 g^{-1} FW) and malondialdehyde (MDA) (nM g^{-1} FW) in bean *(Phaseolus vulgaris* L.) plants grown under three light conditions. Each value is the mean of 4 repetitions ± SD. Bars denoted with the same letter are not significantly different (P<0.05).

effect of Mn treatment was much less pronounced. Increasing light intensity resulted in an accumulation of H_2O_2 in leaves in tendency or signihcant, Cu and Mn treatment caused also further accumulation, Mn being effective than Cu. Similar changes were observed for MDA content of leaves. As expected, light intensity caused a signihcant increase in MDA content of leaves, however, in contrast to Mn, Cu did not affect MDA content of leaves (Fig. 4).

Total amino acids and protein concentration

Content of total amino acids of leaves increased in response to higher light intensity and both of studied heavy metals. Protein concentration, in contrast, remained unchanged in Cu and Mn treated plants compared to control. Growing under intermediate (500 PPFD) and high (800 PPFD) light intensities caused a rise of protein concentration compared to control (Fig. 5).

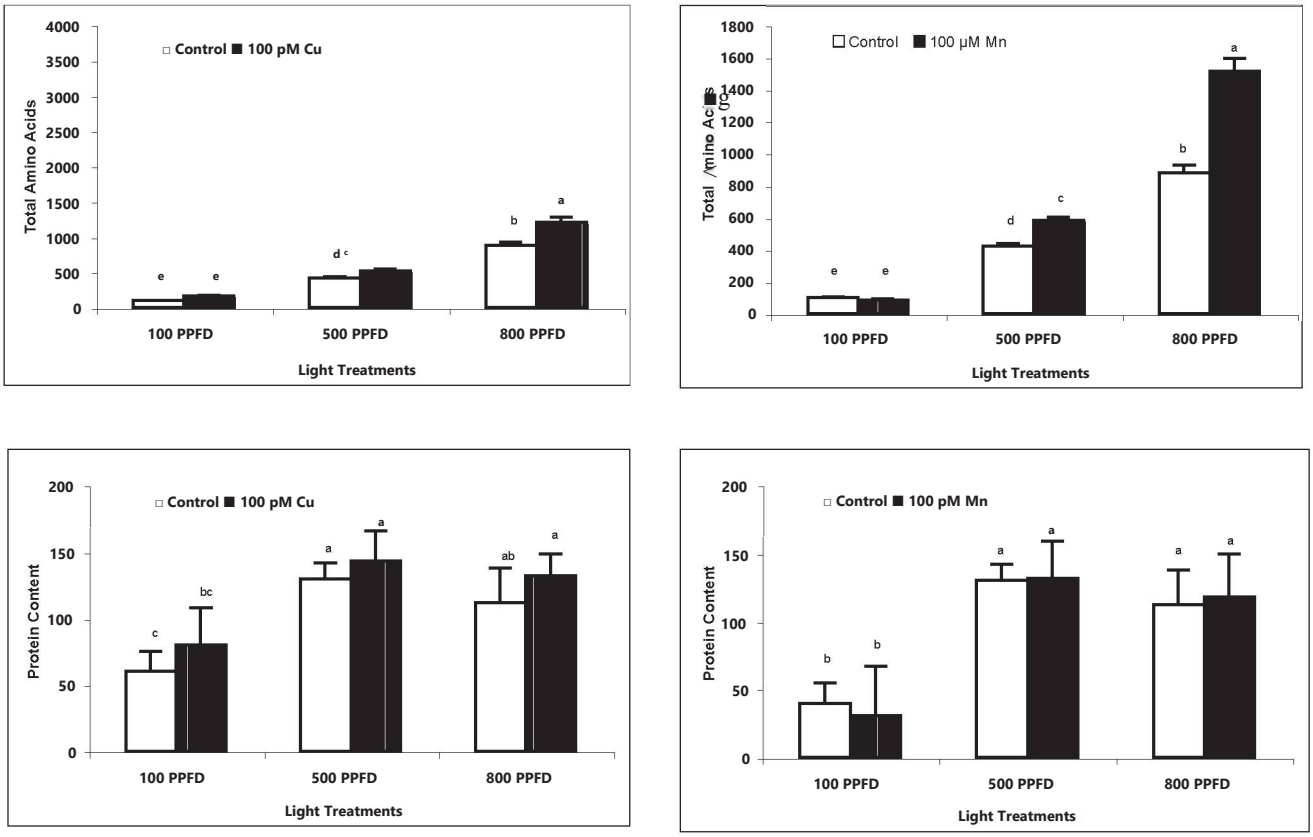

Figure 5. Effect of Cu and Mn treatment on the concentration of total amino acids (|jg g^{-1} FW) and protein (mg g^{-1} FW) in bean *(Phaseolus vulgaris* L.) plants grown under three light conditions. Each value is the mean of 4 repetitions ± SD. Bars denoted with the same letter are not significantly different (P<0.05).

Discussion

Cu toxicity inhibited growth of plants much more than similar concentrations of Mn in the medium. A high susceptibility of plants to Cu toxicity compared to other heavy metals such as Mn and Zn was reported by other authors for other plants species (Marschner 1995). Growing under higher light intensity caused a signihcant rise of heavy metal accumulation likely due to higher transpiration. The rate of transpiration of HL leaves was 2.5 and 2.0 times higher than LL ones for Cu and Mn treated plants respectively.

Leaf chlorophyll a+b concentration, with the exception of an increase at 50 pM Cu in LL plants, did not affect significantly by excess Cu in the medium. In contrast, Mn treatment caused an increase of chlorophyll a+b at all light conditions, showing higher tolerance of leaf chlorophyll to Mn than leaf fresh weight and a concentration effect of chlorophyll. The ratio of chlorophyll a/b was changed by Cu and Mn differently. Considering fresh weight changes and individual values of chlorophyll a and b, it could be shown that degradation of chlorophyll b in response to Cu was greater than growth reduction of leaves, but degradation of chlorophyll a was less than growth impairment. The opposite was observed for Mn. The cause of a higher sensitivity of chlorophyll b to Cu toxicity and of chlorophyll a to Mn toxicity is not known. A higher chlorophyll b degradation than chlorophyll a under Cu stress was observed also in rice and sunflower (Hajiboland and Hasani 2007) and bean (Pat§ikka et al. 2002).

Reduction of assimilation rate in Cu treated plants under higher light intensity was only partly attributable to the reduction of stomatal conductance. Stomatal conductance diminished slightly (15%), while reduction of *A* was much higher and reached up to 71% compared to control in Cu treated HL plants. Greater reduction of *A* in response to Cu under higher light intensity could be attributed to the induction of oxidative stress. It seems most likely that, a combination of two factors i.e. Cu treatment (Luna et al. 1994) and higher light intensity (Behera and Choudhury 2002) both inducing oxidative damage to the cell and particularly photosynthetic membranes (Elefteriou and Karatagalis 1989), inhibit the net assimilation rate much more than that imposed by closure of stomata. Reduction of APX activity by increasing light intensity could be another causes of more reduction of photosynthesis in HL than LL plants in response to toxic concen-

trations of Cu. Limitation in antioxidant capacity due to Cu treatment prevents proper detoxification of reactive oxygen species, which were produced up to great amounts in plants treated by Cu and grown under high light intensity.

However, in Mn treated leaves under all three light conditions the extent of reduction of A was higher than that of g_s, indicating that other factors involve in the reduction of net assimilation rate than combination effect of light and heavy metal. In addition, changes of APX activity could not explain higher sensitivity of net assimilation rate than stomatal conductance to Mn treatment. Though activity of APX decreased in response to higher light intensity, increased in plants due to exposure to Mn. More inhibition of photochemistry of plants under Mn treatment could be of involving factors of decreased net assimilation rate (see below).

The biophysical basis underlying changes in the photosynthetic characteristics of bean plants was assessed using chlorophyll fluorescence. Measurement of chlorophyll a fluorescence is a non-invasive, powerful and reliable method, to assess the PSII function of Cu and Mn treated leaves in this work.

The initial chlorophyll fluorescence yield, F_g reflects the minimal fluorescence yield when all Q_A are in the oxidized state. According to our results, the increase of F_g recorded under Cu and Mn toxicity, can be interpreted as a reduction of the rate of energy trapping by PSII centers and this could be the result of a physical dissociation of light harvesting complex from PSII core, as it has been observed under other stress such as heat (Armond et al. 1980). The increase of F_g value, was higher in Mn compared to Cu treated leaves, therefore, Mn was more effective in reduction of rate of energy trapping by PSII centers. In addition, Cu and Mn treated bean plants had a significant smaller F_m value compared to the control, which in relation to the slightly reduced T_m, indicates that an increasing fraction of reaction centers becomes inactivated. It is well known that when F_m is attained, all PQ molecules are reduced. Thus, we can suggest that F_m depression may reflect also a decreased size of antennas and/or a diminished pool of PQ (Ouzounidou et al. 1998). Moreover, reduction of F_m values in heavy metal treated plants was observed only in HL plants. These values remained unchanged in IL and increased in LL plants. Therefore, a decreased size of antennas and/or a diminished pool of PQ occurred only under the dual effect of heavy metal stress and high light intensity.

Manganese treated plants had higher reduction of F_v than Cu treated ones, indicating that Mn exerted higher inhibitory effect on photochemistry of leaves. Decrease in F_v indicates a reduction in the number of open PSII units and could be attributed to a structural and functional disorder of the photosynthetic apparatus and damage to the PSII (Osmond 1994; Pereira et al. 2000; Murkowski 2001).

The direct effect of increasing F_g, is the slight decrease of F_v/F_m ratio. The maximal quantum yield of PSII (F_v/F_m) ratio declined less than 10% between the control and heavy metal treated leaves. The preservation of F/F_m ratio under Cu and Mn toxicity, as indicated by very small changes, probably is the consequence of a modification of the Q_A to Q_B electron transfer (Ouzounidou et al. 1998). In contrast to F_v/F_m, F_v/F_g ratio decreased significantly in response to heavy metals. The change in the ratio of F_v/F_g was higher in Mn-compared to Cu-treated plants, nevertheless, it was not coincided on differential growth response of plants concerning Cu and Mn treatment.

A higher inhibitory effect of Mn on biophysical processes of photosynthesis could be attributed to the preferential entrance of Mn into chloroplasts and damaging photosynthesis membrane and apparatus. According to the concentration of H_20_2 and MDA as an oxidant and a product of lipid peroxidation respectively, Mn produced more oxidative stress than Cu.

The increasing amount of F_v/F_m in response to increasing light intensity and the high amounts of 0.80-0.83 at HL conditions showed that an enough photochemistry occurred only in HL plants in growth chamber. Therefore, it is expected no photochemical damage occurred even at HL conditions in control plants.

Both of high leaf concentration of heavy metals (Dietz et al. 1999) and high light intensity (Asada 1999) induce an oxidative stress. In this work, activity of CAT, SOD and GR were similarly affected by Cu and Mn treatments. It indicated that, they could not explain different growth response to Cu and Mn. However, results imply that they play an important role in adaptation of plants under dual effect of high light intensity and heavy metal stress.

Activity of APX in Mn treated plants particularly under IL and HL conditions, was induced while in Cu treated ones rather inhibited. Considering that, this difference was coincided on different growth response of plants to Cu and Mn, it could be suggested that APX activity play an important role in Mn resistance of bean plants particularly under higher light intensities.

Activity of POD remained unchanged in Mn treated plants and increased in Cu treated ones. Therefore, activity of POD reflected plants performance under heavy metal stress and changes were coincided on the differential effect of Cu and Mn toxicity in bean plants. (Two lines were removed). In many plant species, excessive uptake of heavy metals such as Ni, Pb and Cd induces a strong increase of peroxidase activities and qualitative changes to their isozyme patterns (Chaoui et al. 1997; Mazhoudi et al. 1997; Soudek et al. 1998; Baccouch et al. 2001). In the present work, the unspecific POD activity assayed with guaiacol as a universal substrate (Gross et al. 1977) was considered as total activity. Guaiacol POD can exhibit activity of APX (antioxidant enzyme), coniferyl alcohol peroxidase (lignifying enzyme), NADH oxidase and IAA oxidase (growth limiting peroxidases). The individual

activity of these enzymes with the exception of APX, were not distinguished from the soluble pool in our extraction procedure. The effects of heavy metals on the activity of oxygen radical detoxifying peroxidases and their involvement in the defense mechanisms of plant tissues against metal-induced damages have been widely reported, but remain controversial (Van Assche and Clijsters 1990; Chaoui et al. 1997; Mazhoudi et al. 1997; Weckx and Clijsters 1997; Cuypers et al. 2000).

Similar to the activity of POD, accumulation of proline was also mainly associated with stress conditions asjudged by growth data. Higher light intensity did not affect proline concentration in control plants but caused an accumulation of proline in Cu and Mn treated plants. Copper was most effective in induction of proline accumulation than Mn under all light conditions. Proline accumulation accepted as an indicator of environmental stresses, is also considered to have important protective roles (Castillo 1992). According to antioxidant activity and chelating properties, accumulation of proline in this work should results in higher tolerance of bean plants to Cu toxicity compared to Mn. However, similar to POD activity, accumulation of proline monitored the stress conditions, but did not caused necessarily more protection against and higher adaptation to Cu compared to Mn in bean plants.

Conclusion

In this work, gas exchange parameters of photosynthesis were affected similarly by Mn and Cu. In contrast, biophysical processes of photosynthesis as judged by various chlorophyll fluorescence data, were more sensitive to Mn than Cu. Therefore, growth responses to Cu and Mn were reflected neither by the chlorophyll fluorescence parameters nor the net assimilation rate. However, the remarkable reduction of total plant leaf area could affect whole plant photosynthesis, contributing to the low biomass production of Cu compared to Mn treated plants.

From enzymes studied, only APX showed a role in the Mn tolerance of plants under higher light conditions. But unexpectedly, it was not associated with higher protection of photochemistry of Mn treated plants. On the other hand, concentration of H_20_2 as an oxidant and MDA as the product of lipid peroxidation were higher in Mn than Cu treated plants, indicating a higher oxidative stress despite of a higher growth in the former than latter plants. Therefore, other biochemical and physiological processes should be involved in the plant performance under heavy metal stress as well as different response to Cu and Mn in bean plants.

Plants have a range of potential mechanisms at the cellular level that might be involved in the detoxification and thus tolerance to heavy metal stress. These all appear to be involved primarily in avoiding the build-up of toxic concentrations at sensitive sites within the cell and thus preventing the damaging effects described above, rather than, developing proteins that can resist the heavy metal effects. Thus, for example, there is little evidence that tolerant species or ecotypes show an enhanced oxidative defense, rather tolerant plants show enhanced avoidance and homeostatic mechanisms to prevent the onset of stress. In this work, Cu induced accumulation of proline may have an important role in the chelation and detoxification of Cu, which resulted in lower H_20_2 accumulation and lipid peroxidation, even though it did not results in higher resistance for bean plants comparing to the effect of Mn. It can be suggested that the antioxidant capacity of plants could not be independently correlated with the growth response of plants without regarding the other physiological responses to metal exposure. It has been suggested that the tolerance to metal toxicity is more dependent on the availability of reduced cell metabolites, than on antioxidative enzymes capacity of plant tissues (Cuypers et al. 2000; Léon et al. 2002; Chaoui et al. 2004). On the other hand, deleterious effects of heavy metal stress in plants may be coupled to other physiological processes via the stimulation of some enzymatic activities that limit cell growth.

References

Alia P, Saradhi PP (1991) Proline accumulation under heavy metal stress. J. Plant Physiol 138:554-558.

Armond PA, Bjorkman O, Staehlin LA (1980) Dissociation of supra-molecular complexes in chloroplast membranes. A manifestation of heat damage to the photosynthetic apparatus. Biochem Biophys Acta 601:433-442.

Asada K (1999) The water-water cycle in chloroplasts: scavenging of active oxygen and dissipation of excess photons. Ann Rev Plant Physiol Plant Mol Biol 50: 601-639.

Baccouch S, Chaoui A, El Ferjani E (2001) Nickel toxicity induces oxidative damage in *Zea mays* Roots. J Plant Nutr 24(7):1085-1097.

Bates LS, Waldren SP Teare ID (1973) Rapid determination of free proline for water-stress studies. Plant Soil 39:205-207.

Behera RK, Choudhury NK (2002) High irradiance induced pigment degradation and loss of photochemical activity of wheat chloroplasts. Biol Plant 45(l):45-49.

Boominathan R, Doran P (2002) Ni-induced oxidative stress in roots of the Ni hyperaccumulator, *Alyssum bertolonii*. New Phytol 156:205-215.

Bradford MM (1976) A rapid and sensitive method for the Quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 72:248-254.

Bridger GM, Yang W, Falk DE, Mc Kersie BD (1994) Cold acclimation increases tolerance of activated oxygen in winter cereals. J Plant Physiol 144:235-240.

Castillo FJ (1992) Peroxidases and stress, plant peroxidases 1980-1990. In Penel C, Gaspar T, Greppin H, eds., Topics and Detailed Literature on Molecular, Biochemical, Physiological Aspects. University of Geneva Press, Geneva, 187-203.

Chance B, Maehly AC (1955) Assay of catalases and peroxidases. Methods in Enzymology 2: 764-775.

Chaoui A, Jarrar B, El. Ferjani E (2004) Effects of cadmium and copper on peroxidas, NADH oxidase and IAA oxidase activities in cell wall, soluble and microsomal membrane fractions of pea roots. J Plant Physiol 161:1225-1234.

Chaoui A, Mazhoudi S, Ghorbal MH, El Ferjeni E (1997) Cadmium and zinc induction of lipid peroxidation and effects on antioxidant enzyme activities in Bean (*Phaseoulus vulgaris* L.). Plant Sci 127:139-147.

Chen L-M, Lin CC, Kao CH (2000) Copper toxicity in rice seedlings: changes in antioxidative enzyme activities, H_20_2 level, and cell wall peroxidase activity in roots. Acta Bot Sin 41: 99-103.

Costa G, Morel J-L (1994) Water relations, gas exchange and amino acid content in Cd-treated lettuce. Plant Physiol Biochem 32: 561-570.

Cuypers A, Vangronsveld J, Clijsters H (2000) Biphasic effect of copper on the ascorbate-glutathione pathway in primary leaves of ***Phaseolus vulgaris*** L. seedlings during the early stages of metal assimilation. Physiol Plant 110:512-517.

De Vos CHR, Schat H (1991) Free radicals and heavy metal tolerance. In Rozeman J, Verkleij JAC eds., Ecological Responses to Environmental Stresses. Kluwer Academic Publishers, Dordrecht, The Netherlands, 22-30.

Dietz KJ, Baier M, Kramer U (1999) Free radicals and reactive oxygen species as mediators of heavy metal toxicity in plants. In Prasad MNV, Hagemeyer J, eds., Heavy Metal Stress in Plants: From Molecules to Ecosystems. Springer-Verlag, Berlin, 73-97.

Elefteriou EP, Karatagalis S (1989) Ultrastrnctural and morphological characteristics of cultivated wheat growing on copper-polluted fields. Bot Acta 102:134-140.

Farago ME, Mullen WA (1979) Plants which accumulate metals. IV. A possible copper-proline complex from the Roots of ***Armería*** *maritime*. Inorg Chem Acta 32:L93-L94.

Fernandes JC, Henriques FS (1991) Biochemical, physiological and structural effects of excess copper in plants. Bot Rev 57:246-273.

Foy CD, Chaney RL, White MC (1978) The physiology of metal toxicity in plants. Ann Rev Plant Physiol 29:511-566.

Foyer CH, Halliwell B (1976) The presence of glutathione and glutathione reductase in chloroplasts: A proposed role in ascorbic acid metabolism. Planta 133:21-25.

Gaspar T, Penel C, Castillo FJ, Greppin H (1984) A two step control of basic and acidic peroxidases and its significance for growth and development. Physiol Plant 64:418-423.

Giannopolitis CN, Ries SK (1977) Superoxide dismutase I. Occurrence in higher plants. Plant Physiol 59:309-314.

González A, Steffen KL, Lynch P J (1998) Light and excess manganese. Implication for oxidative stress in common bean. Plant Physiol 18:493-504.

Gounaries K, Barber J, Harwood JL (1986) The thylakoid membranes of higherplant chloroplasts. Biochem J 237:313-326.

Gressel J, Galun E (1994) Genetic controls of photooxidant tolerance. In Loyer CH, Mullineaux P, eds., Causes of Photooxidative Stress and Ameliration of Defence Systems in Plants. CRC Press, Boca Raton, Florida, 237-273.

Gross GG, Janse C, Elstner EF (1977) Involvement of malate, monophenols and the superoxide radical in hydrogen peroxide formation by isolated cell walls from horseradish ***(Armoracia lapathifolia*** Gilib.). Planta 136:271-276.

Hajiboland R, Hasani BD (2007) Effect of Cu and Mn toxicity on chlorophyll fluorescence and gas exchange in rice and sunflower under different light intensities. J Stress Physiol Biochem 3(1):4-17.

Hall JL (1994) Cellular Mechanisms for heavy metal detoxification and tolerance. J Exp Bot53 (366):1-11.

Heath RL, Packer L (1968) Photoperoxidation in isolated chloroplasts I. Kinetics and stoichiometry of fatty acid peroxidation. Arch Biochem Biophys 25:189-198.

Horiguchi T (1988) Mechanism of manganese toxicity and tolerance of plants. VII. Effect of light intensity on manganese-induced chlorosis. J Plant Nutr 11: 235-245.

Hwang M, Ederer GM (1975) Rapid hippurate hydrolysis method for presumptive identification of group B streptococci. J Cli Microbiol 1:114.

Karataglis S, Moustakas M, Symeonidis L (1991) Effects of heavy metals on isoperoxidases of wheat. Biol Plant (Praha) 33:3-9.

Kautsky H, Appel W, Amann H (I960) Chlorophyllfluorescenzund Kohlensaureassimilation. Biochemishe Zeitschrift 322:277-292.

Kyle DJ (1987) The biochemical basis for photoinhibition of photosystem II. In Kyle DJ, Osmond CB, Arntzen CJ, eds., Photoinhibition. Elsevier, Amsterdam, 197-226.

Léon AM, Palma JM, Corpas FJ, Comez M, Romero-Puertas MC, Chatterjee D, Mateos RM, del Rio LA, Sandalio LM (2002) Antioxidative enzymes in cultivars of pepper plants with different sensitivity of cadmium. Plant Physiol Biochem 40:813-820.

Luna CM, Gonzalez CA, Tripp VS (1994) Oxidative damage caused by excess of copper in oat leaves. Plant Cell Physiol 35:11-15.

Marschner H (1995) Mineral Nutrition of Higher Plants. 2nd Edition, Academic Press, UK.

Maxwell K, Johnson GN (2000) Chlorophyll fluorescence- A practical guide. JExpBot51(345):659-668.

Mazhoudi S, Chaoui A, Ghorbal MH, El Ferjani E (1997) Response of antioxidant enzymes to excess copper in tomato ***(Lycopersicon esculentum*** Mill.). Plant Sei 127:129-137.

Me Cain DC, Markley JL (1989) More manganese accumulates in maple sun leaves than in shade leaves. Plant Physiol 90:1417-1421.

Mocquot B, Vangronsveld J, Clijsters H, Mench M (1996) Copper toxicity in young maize (Zea mays L.) plants: Effects on growth, mineral and chlorophyll contents, and Eezyme activities. Plant Soil 128:287-300.

Moran R (1982) Formulae for determination of chlorophyllous pigments extracted with N,N-dimethylforamide. Plant Physiol 69:1376-1381.

Murkowski A (2001) Heat stress and spermidine, effect on chlorophyll fluorescence in tomato Plants. Biol Plant 44:53-57.

Nable RO, Houtz RL, Cheniae GM (1988) Early inhibition of photosynthesis during development of mn toxicity in tobacco. Plant Physiol 86:1136-1142.

Neumann G, Massonneau A, Martinoia E, Römheld V (1999) Physiological adaptations to phosphorus deficiency during proteoid root development in white lupin. Planta 208:373-382.

Osmond CB (1994) What is photoinhibition? Some insights from comparisons of shade and sun plants. In Baker N., Bowyer JR, eds., Photoinhibition of Photosynthesis, from Molecular Mechanism to the Field. Bios Scientific Publisher, Oxford, UK, 1-24.

Ouzounidou G, Ilias I, Tranopoulou H, Karatagalis S (1998) Amelioration of copper toxicity by iron on spinach Physiology. J Plant Nutr 21:2089-2101.

Pätsikkä E, Aro EM, Tyystjärvi E (1998) Increase in the quantum yield of photoinhibition contributes to copper toxicity ***in*** vivo. Plant Physiol 117:619-627.

Pätsikkä E, Aro EM, Tyystjärvi E (2001) Mechanism of copper-enhanced photoinhibition in thylakoid membranes. Physiol Plant 113:142-150.

Pätsikkä E, Kairavuo M, Šeršen F, Aro EM, Tyystjärvi E (2002) Excess copper predisposes photosystem II to photoinhibition ***in*** vivo by outcompeting iron and causing decrease in leaf chlorophyll. Plant Physiol 129:1359-1367.

Patterson BD, Mac Rae EA, Ferguson IB (1984) Estimation of hydrogen peroxide in plant extracts using titanium (IV). Anal Biochem 139:487-492.

Pereira WE, de Siqueira DL, Martinez CA, Puiatti M (2000) Gas exchange and chlorophyll fluorescence in four citrus rootstocks under aluminum stress. J Plant Physiol 157:513-520.

Schat H, Sharma SS, Vooijs R (1997) Heavy metal-induced accumulation of free proline in a metal tolerant and a non-tolerant ecotype of ***Silene vulgavulgaris***. Physiol Plant 101:477-482.

Schöner S, Krause GH (1990) Protective systems against active oxygen species in spinach: Response to cold acclimation in excess light. Planta 180:383-389.

Simon LM, Fatrai Z, Jonas DE, Matkovics B (1974) Study of peroxide metabolism enzymes during the development of ***Phaseolus vulgaris.*** Biochem Physiol Pflanzen (BPP) 166:387-392.

Smirnoff N, Cumbes QJ (1989) Hydroxy radical scavenging activity of compatible solutes. Phytochemistry 28:1057-1060.

Soudek P, Podlipná R, Lipavská H, Vaněk T (1998) Bioaccumulation of heavy metals by hairy root culture of ***Armoratia rusticana*** L. Pharm Paharmacol Lett 8:57-60.

Van Assche F, Clijsters H (1990) Effects of metals on enzyme activity in plants. Cell and Environ 13:195-206.

Vichnevetskaia KD, Roy DN (1999) Oxidative stress and antioxidative defense with an emphasis on plants antioxidants. Environ. Rev7:31-51.

Weckx JEJ, Clijsters, HMM (1997) Zn phytotoxicity induces oxidative stress in primary leaves of ***Phaseolus vulgaris.*** Plant Physiol Biochem 35:405-410.

Wissemeier AH, Horst WJ (1992) Effect of light intensity on manganese toxicity symptoms and callose formation in cowpea (*Vigna* ***unguiculata*** L. Walp.). Plant Soil 143:299-309.

Woolhouse HW (1983) Toxicity and tolerance in the responses of plants to metals. In Lange OL, Nobel PS, Osmond CB, Ziegler H, eds., Encyclopedia of Plant Physiology, Vol 12C: Physiological Plant Ecology III. Springer Verlag, Berlin, Germany, 245-300.

Yrnela I, Gatzen G, Picorel R, Holzwarth AR (1996) Cu (II)-inhibitory effect on photosystem II from higher plants: A picosecond time-resolved fluorescence study. Biochemistry 35:9469-9474.

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ARTICLE

Cytogenetic study of some *Hordeum* L. species in Iran

Masoud Sheidai*, Samaneh Rashid

Faculty of Biological Sciences, Shahid Beheshti University, Tehran, Iran

ABSTRACT Karyotypic and meiotic studies were performed in 17 populations of seven *Hordeum* species and subspecies showing the occurrence of 2n = 2x = 14, 2n = 4x = 28 and 2n = 6x = 42. Tetraploid level for *H. distichon* and hexaploid level for *H. leporinum* are new reports. The chromosomes were mostly metacentric and sub-metacentric. Significant differences in the size of chromosomes and their arm ratios indicated the occurrence of quantitative changes in the chromatin (DNA) material during species diversification. The species also differed in their karyotypic formulae possibly due to the occurrence of chromosome structural changes. Comparison of the total haploid chromatin length among populations of *H. bulbosum* and *H. glaucum* having diploid and tetraploid chromosome numbers, indicated that the occurrence of polyploidy is not associated with a significant increase in the amount of DNA and almost the same DNA content has been distributed among the chromosomes of the tetraploid population. The relative karyotypic data were used for multivariate analysis, which showed karyotypic variations among *Hordeum* species and populations studied. Acta Biol Szeged 51(2):107-112 (2007)

KEY WORDS

Cytogenetic
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The genus *Hordeum* L. (barley) of the family Pooideae (subfamily Hordeae) is comprised of about 40 annual or perennial species including diploid *(In* = 2x = 14) and polyploid *(In* = 4x = 28, *2n = 6x* = 42) species or cytotypes (Bothmer et al. 1995), mainly distributed in the North temperate and South America but also available in South-West and central Asia. The *Hordeum* are commonly adventive, mesophytic, or xerophytic species of open habitats; halophytic and glycophytic. They usually grow in open weedy or sandy places and mostly in dry soils (Bothmer et al. 1991; Shewry 1992, Watson and Dallwitz 1992). The genus *Hordeum* possesses some signihcant weed species like *H. jubatum, H. leporinum, H. marinum, H. murinum* (the fruiting inflorescence parts of this causing eye and other damage to livestock, and problems with wool) and some grain crop species including *H. vulgare* (Barley).

The genus originated about 12 MYA (Blattner 2004) in western Eurasia from where it colonizedits extant distribution area in Europe, central Asia, North America, South America, and South Africa. The monophyly of the genus is well supported by morphological (Sebergand Frederiksen 2001) and molecular phylogenetic studies (Petersen and Seberg 1997; Blattner 2004), whereas the intrageneric phylogenyis still a matter of dispute (Sabine and Blattner 2006).

The species of *Hordeum* growing in Iran varies according to different authors, Parsa (1950), Mobayyen (1981) and Bor (1970) reported the occurrence of 11 *Hordeum* species in Iran, but the species they report vary from each other. Moreover, Termeh (1986) reported *H. bogdanii* from Iran which is not reported by the previous authors. These species grow wild mostly in the north, west-north and south-west of Iran and are considered as important forage plants of the country.

According to Flora Iranica (Bor 1970), the *Hordeum* species of Iran, are distributed in *4* different sections of 1- *Bulbohordeum,* 2- *Crithe,* 3- *Hordeastrum* and *4- Stenostachys.* The present study considers karyotypic details of 14 populations of 7 *Hordeum* species and subspecies as well as meiotic analysis of 3 populations of two species for the first time.

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Corresponding author. E-mail: msheidai@sbu.ac.ir

Materials and Methods

Plant materials

Karyotypic studies were performed on 14 populations of 6 *Hordeum* species and 1 sub-species namely: 1- *H. bulbosum* L. (two populations), from the section *Bulbohordeum,* 2- *H. distichon* L., 3- *H. spontaneum* C. Koch (two populations) and 4- *H. vulgare* L. (two populations), from the section *Crithe,* 5- *H. glaucum* Steud. (five populations), 6- *H. leporinum* Link., and 7- *H. marinum* Huds. subsp. *Marinum,* from the section *Hordeastrum,* while the meiotic studies were performed on 3 populations of *H. bulbosum* and *H. spontaneum.*

Cytological studies

For karyotypic studies freshly grown root tips were collected from the seeds of at least 10 randomly selected plants in each species, pretreated with 0.002 mol 8-hydroxyquinolin (1-2 hrs.) and fixed in ethanol: acetic acid (3:1) for 24 hrs. The

Table **1.** Karyotypic details of *Hordeum* species and populations studied.

A2	A,	ST	TF%	No. sat	Sat.ch	S	L	T.L	2n	Polidy level	Locality	Sp. code	Species
16.49	0.26	1A	41.90	-	-	13.75	20.5	34.25	14	2x	Tehran	H.b1	*H. bulbosum*
18.04a	0.32	2A	40.13	2	11,14	12.2	23.4	35.6	28	4x	Arasbaran	H.b2	*H. bulbosum*
16.75	0.29	2A	40.34	3	1, 2, 3	14.49	22.87	37.36	14	2x	Dorood	H.d	*H. distichon*
16.49	0.31	2A	40.20	2	3,6	12	20.25	32.25	14	2x	Sangsefid	H.g1	*H. glaucum*
25.03	0.32	2B	39.27	1	1	8.2	18.5	27.32	14	2x	Sabzevar	H.g2	*H. glaucum*
24.49	0.28	1B	42.09	1	1	11.74	24	35.75	14	2x	Ardebil	H.g3	*H. glaucum*
19.09	0.36	2A	38.75	5	1,3,4,5,7	11.35	20.65	32	14	2x	Abadan	H.g4	*H. glaucum*
19.60	0.44	2A	35.09	-	-	13	23.25	36.25	28	4X	Tehran	H.g5	*H. glaucum*
22.97	0.30	2B	40.11	3	11,13,15	11	23.25	24.25	42	6X	Shazand	H.l	*H. leporinum*
20.64	0.36	2A	38.25	2	5,7	10.25	19.18	29.43	14	2x	Abadan	H.m	*H. marinum ssp. marinum*
16.04	0.34	1A	39.28	3	1,6,7	12.93	20.20	32.95	14	2x	Tehran	H.s1	*H. spontaneum*
16.54	0.27	1A	41.97	2	5,7	12.49	19.33	31.82	14	2x	Darake	H.s2	*H. spontaneum*
25.96	0.36	1B	38.86	5	2,4,5,6,7	14.55	31.7	46.25	14	2x	Ahvaz	H.v1	*H. vulgäre*
10.87	0.36	1A	38.86	2	3,5	16.66	22.83	39.49	14	2x	Abadan	H.v2	*H. vulgäre*

TL = Total haploid chromatin length (pm), L = Longest chromosome (pm), S = Shortest chromosome (pm), Sat = Sat-chromosome, TF = Total form percentage, ST = Stebbins class, A_1 ,A_2 = Romero-Zarco indices.

fixed tips were then washed thoroughly in distilled water and macerated in 60°C IN HC1 for about 30 seconds. Squash technique was used for cytological studies with 2% aqueous aceo-orcein as the stain. The somatic chromosome number and karyotypic details were studied in at least 5 well-prepared metaphase plates. The chromosomes were sketched with the use of a Camera Lucida and measurements were performed accordingly from such sketches. The chromosomes were identified according to Levan et al. (1964), karyotype symmetry was determined according to Stebbins (1971) and A1 and A2 symmetry indices of Romero-Zarco (1986), while other karyotypic parameters like total form percentage (TF%) and coefficient of variation of the chromosome size were also determined (Sheidai et al. 2000).

Young flower buds were collected from 10 randomly selected plants of each species/ population and fixed in glacial acetic acid: ethanol (1:3) for 24 hrs. Flower buds were washed and preserved in 70% ethanol at 4°C until used (Sheidai et al. 2002, 2003). Cytological preparations used squash technique and 2% aceto-orcein as the stain.

Fifty to one hundred pollen mother cells (PMCs) were analysed for chiasma frequency and distribution at diakinesis. metaphase stage and 500 PMCs were analysed for chromosome segregation during the anaphase and telophase stages.

Statistical analyses

The analysis of variance (ANOVA) and the least significant difference test (LSD) were performed to reveal significant differences in the size of chromosomes among the populations of each species as well as among species with similar somatic chromosome numbers (Sheidai et al. 2000). In order to group the species studied based on similarity in their karyotypic features different clustering methods of UPGMA (Unweighted Paired Group with Arithmetic Average) and WARD (minimum spherical cluster method) as well as ordination based on principal coordinate analysis (PCO) were performed. Since the species studied possess different somatic chromosome numbers, relative karyotypic and meiotic parameters were used in clustering and PCO ordination (Table 1). NTSYS Ver. 2.02 (1998) was used for clustering and PCO analyses.

Results and discussion

The somatic chromosome number and karyotypic details of the *Hordeum* species and populations studied are presented in Table 1 and Figs. 1 & 2. Tehran population of *H. bulbosum* possessed 2n = 2x = 14 while, Arasbaran population possessed 2n = 4x = 28 chromosome number supporting the earlier reports (Strid andFranzen 1981; Semenov 1986). Dorood population of *H. distichon* possessed 2n = 2x = 14 chromosome number, supporting the earlier report (Zhang et al. 1990). Among 5 populations of *H. glaucum* studied, Tehran population possessed 2n = 4x = 28 chromosome number while, the other populations were diploid. The earlier studies reported only diploid chromosome number for this species (Farugi 1985, Avagian et al. 1989) therefore tetraploid level is new for *H. distichon.*

Shazand population of the species *H. leporinum,* possessed 2n = 6x = 42 chromosome number. The earlier studies reported only diploid and tetraploid chromosome numbers for this species (Hatch 1980; Valdés et al. 1999) therefore hexaploid level is new for *H. leporinum.*

Populations of *H. marinum subsp. marinum, H. spontaneum* and *H. vulgare* possessed 2n = 2x = 14 supporting the earlier reports (Nicoloff et al. 1977; Chen and Wang 1988; Spies et al. 1999). Tetraploid level has been reported for *H. marinum subsp. Marinum* by Avagian et al. (1989). Both

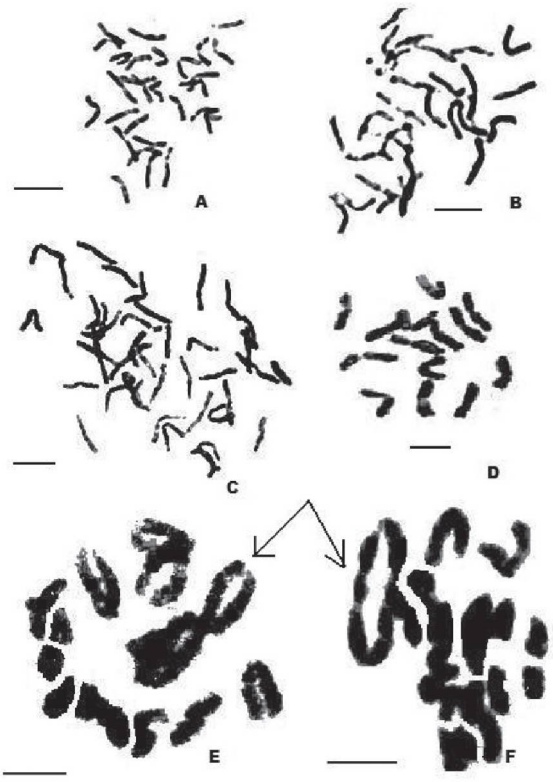

Figure 1. Representative somatic and meiotic cells in *Hordeum* species studied. A-D= Metaphase cells in Arasbaran population of *H. bulbosum* (2n = 28), Tehran population of *H. glaucum* (2n = 28), Shazand population of *H. leporinum* (2n = 42) and Tehran population of *H. spontaneum* (2n = 14). E & F = Meiotic cells in Tehran population of *H .bulbosum* showing quadrivalents (arrow). Scale bar = 10 pm.

tetraploid and hexaploid level have also been reported for *H. vulgare* (Koba 1993; Chen and Wang 1998).

Polyploidy and inter-specibc hybridization is considered to be of high importance in the evolution of Gramineae (Stebbins 1982,1986), the occurrence of different polyploidy levels in different *Hordeum* species and also among populations of a single species, indicates the role of polyploidy in the evolution and adaptation of these species.

The *Hordeum* species and populations studied mainly possessed metacentric (m) and sub-metacentric (sm) chromosomes, however, sub-telocentric chromosomes (st) occurred in Tehran population of *H. glaucumand* Shazand population of *H. leporinum* (Table 1). Variations observed in the karyotypic formulae, the number of SAT-chromosomes and the chromosomes carrying secondary constriction in different populations of each species and also among different species studied, indicate the occurrence of chromosomes structural changes.

The highest value of haploid total chromatin length (46.25 pm) occurred in Ahvaz population of *H. vulgare* which is diploid, while the lowest value of the same (24.25 pm) occurred in hexaploid *H. leporinum,* indicating that the increase in the chromosome number is associated with some degree of DNA loss possibly in the repetitive parts of the genome. Similarly the highest value of the size of longest chromosome (31.70 pm) occurred in Ahvaz population of *H. vulgare,* while the lowest value of the same (18.50 pm) occurred in Sabzevar population of *H. glaucum.*

Comparison of the total haploid chromatin length among two populations of *H. bulbosum* having diploid (34.25 pm) and tetraploid chromosome number (35.60 pm, Table 1), indicates that the occurrence of polyploidy is not associated with a signihcant increase in the amount of DNA and almost the same DNA content has been distributed among the chromosomes of the tetraploid population. A similar situation exists in diploid and tetraploid populations of *H. glaucum* (except for Sabzevar population, Table 1). The ANOVA and LSD tests performed for the size of chromosomes among *Hordeum* species and populations studied, showed a signihcant difference ($p < 0.05$) in the size of chromosomes among different *Hordeum* species but not among populations of a single species. Therefore a signihcant change in DNA content has possibly been associated with the species diversihcation in the genus *Hordeum.*

Genome size variation in plants is thought to be correlated with cytological, physiological, or ecological characters. However, conclusions drawn in several studies are often contradictory (Kalendar et al. 2000). To analyze nuclear genome size evolution in a phylogenetic framework, Blattner (2004), studied DNA contents of 134 accessions, representing all but one species of the genus *Hordeum* by how cytometry. The 2C DNA contents obtained were in a range from 6.85 to 10.67 pg in diploids (2n = 14) and reached up to 29.85 pg in hexaploid species (2n = 42). The smallest genomes were found in taxa from the New World, which became secondarily annual, whereas the largest diploid genomes occur in Eurasian annuals. Genome sizes of polyploid taxa equaled mostly the added sizes of their proposed progenitors or were slightly (1% to 5%) smaller. The analysis of ancestral genome sizes on the base of the phylogeny of the genus revealed lineages with decreasing and with increasing genome sizes. Correlations of intraspecihc genome size variation with the length of vegetation period were found in *H. marinum* populations from Western Europe but were not signihcant within two species from South America. On a higher taxonomical level *(i.e.,* for species groups or the entire genus), environmental correlations were absent. This could mostly be attributed to the superimposition of life-form changes and phylogenetic constraints, which conceal ecogeographical correlations.

The highest value of coefficient of variation (CV) for the size of chromosomes occurred in Ahvaz population of *H. vulgare* (25.96) indicating the highest amount variation in the size of its chromosomes. The lowest value of CV (10.87) occurred in Abadan population of *H.* vulgare.

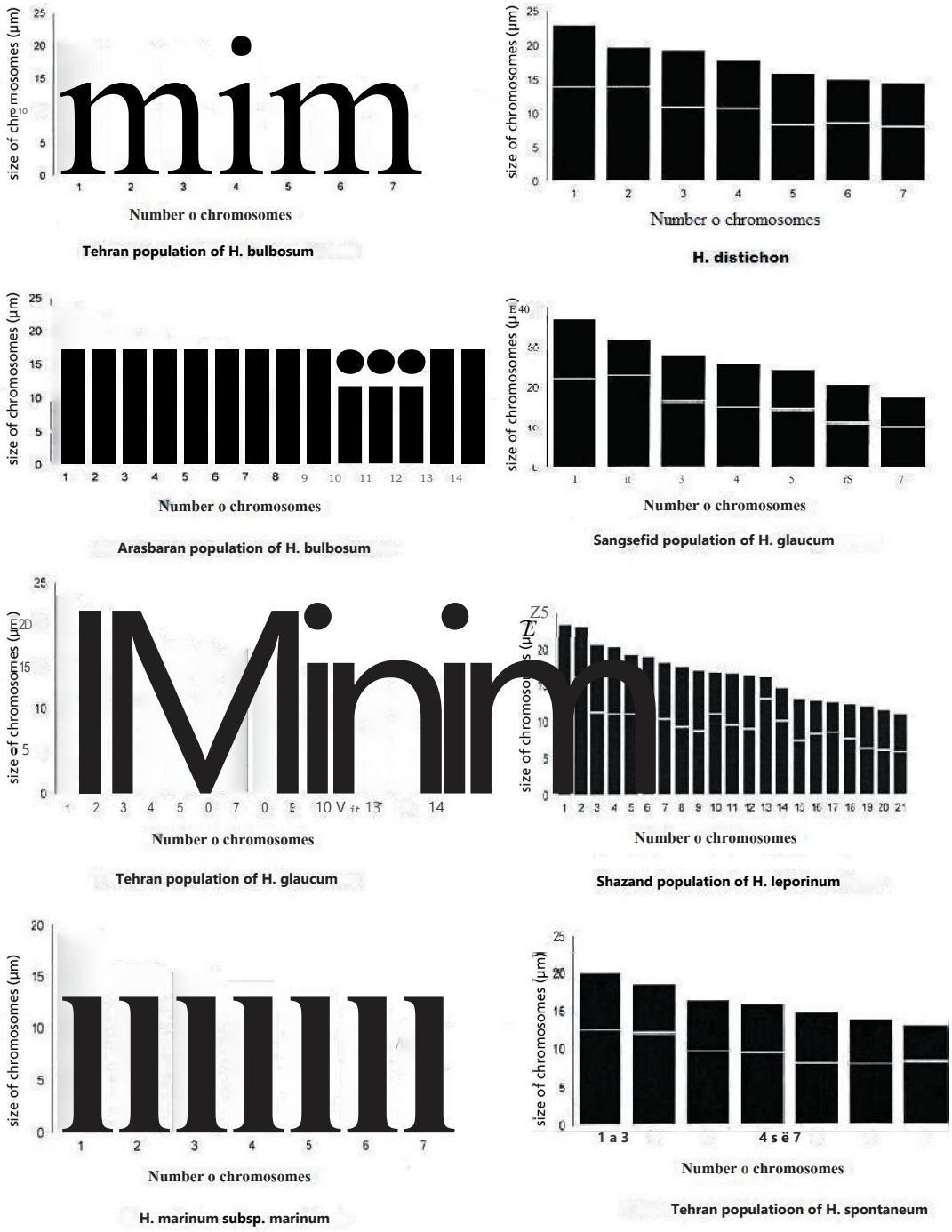

Figure 2. Representative ideograms of *Hordeum* species studied.

The highest value of total form percentage (TF %) occurred in the Ardebil population of *H. glaucum* (42.09) indicating the presence of a symmetrical karyotype, while the lowest value occurred in Tehran population of *H. glaucum* (35.09) having a more asymmetrical karyotype compared to Ardebil population. In terms of the Stebbins two system of karyotype symmetry, the *Hordeum* species studied mostly occupy 1A, IB and 2B classes, which are considered rather primitive classes in this system.

Different populations of the *Hordeum* species studied occupy different classes of karyotype symmetry due to the occurrence of structural changes in their chromosomes (Table

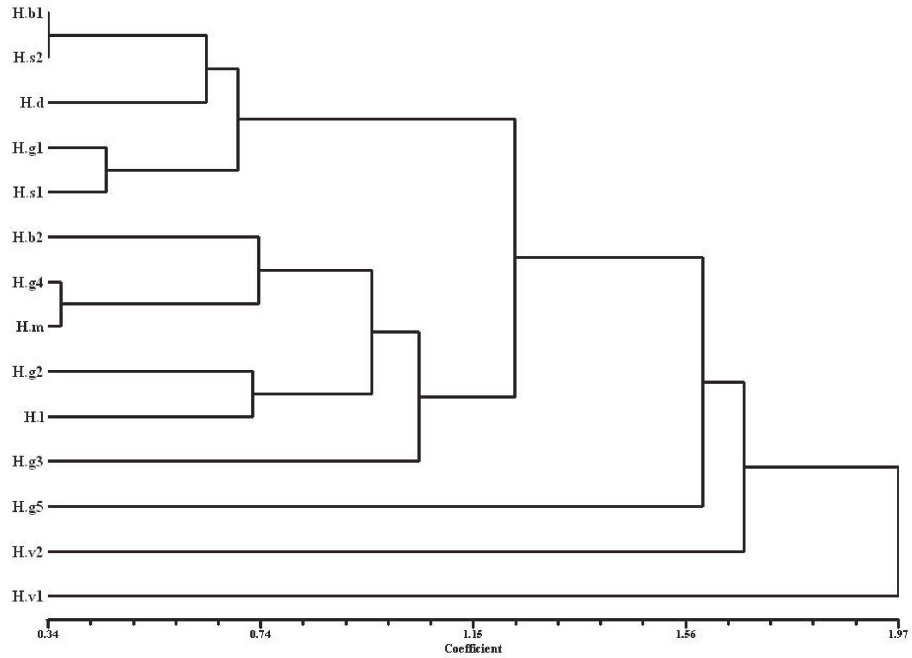

Figure 3. UPGMA clustering of *Hordeum* species studied. (Species code as in Table 1.).

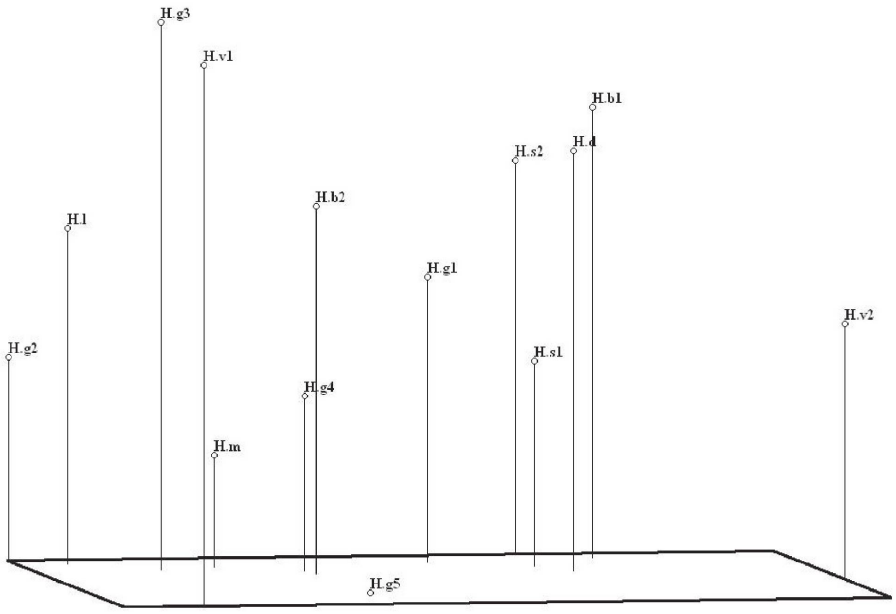

Figure 4. PCO ordination of *Hordeum* species studied. (Species code as in Table 1.).

1). By using the Romero-Zarco symmetry indices of A1 and A2 we can determine the more asymmetric karyotype among the species having similar Stebbins classes of symmetry. For example among the species with the 1A class, Abadan population of *H. vulgare* possesses the highest A1 value (0.36) and a more asymmetric karyotype. Similarly, among the species with the 2A symmetry class, Tehran population of *H. glaucum* possessed the highest value for A1 (0.44) and the highest asymmetric karyotype and among the species with 2B symmetry class, Sabzevar population of *H. glaucum*

possesses the most asymmetric karyotype.

The grouping of the *Hordeum* species and populations based on relative karyotypic data is presented in Figs. 3 & 4. Both UPGMA clustering and PCO ordination produced similar results revealing karyotypic variation among populations of each species, as these populations have been placed in different clusters or groups. For example, Tehran population of *H. bulbosum* is placed in the hrst major cluster while, Arasbaran population of this species has been placed in the second major cluster with some distance from the hrst cluster. The same holds true for different populations of *H. glaucum, H. vulgare* and *H. spontaneum,* revealing the role of chromosomal changes along with polyploidy in the evolution of the *Hordeum* species.

Meiotic analysis

Meiotic studies of 3 populations of *H. bulbosum* and *H. spontaneum,* showed that Tehran and Darake populations of *H. bulbosum* possess 2n = 4x = 28 and *H. spontaneum* possesses 2n = 2x = 14, supporting our karyotypic results. In all populations the chromosomes mainly formed bivalents and in Tehran population of *H. bulbosum* 1-2 ring or chain quadrivalents were formed (Fig. 1). Chromosomes segregation during anaphase was normal in most of the anaphase and telophase cells in all 3 populations, except few cases of laggard chromosomes formation and chromosome stickiness.

References

Avagian IG, Atayeva GM, Romanova AB, Ghandilian PA (1989) To the question on systematic situation of some representatives of the genus *Hordeum* L. Analysis of C-stained chromosomes of taxons of Murina Nevsky series. Biologicheskii Zhurnal Armenii 42:621-629.

Blattner FR (2004) Phylogenetic analysis of Hordeum (Poaceae) as inferred by nuclear. rDNA ITS sequences. Mol Phylogenet Evol 33.289-299.

BoR NL (1970) *Hordeum.* In Reschinger KH, ed. Flora Iranica 70:105-141. Akademische Drnck, Verlagsanstalt, Graz, Austria.

Bothmer R von, Jacobsen N, Baden C, Jorgensen RB, Linde- Laursen I (1991) An ecogeographical study of the genus *Hordeum.* IB PGR (International Board for plant Genetic Resources), Rome.

Bothmer R von, Jacobsen N, Baden C, Jprgensen RB, Linde-Laursen I (1995) An ecogeographical study of the genus *Hordeum*. 2nd ed. Rome, Italy, IPGRI.

Chen, YR, Wang LQ (1988) Morphological and cytogenetical studies on FI of *Hordeum vulgare* L. and *H. bulbosum* L. J Zhejiang Agric 14(2): 142-148.

Farngi SA (1985) Studies of Libyan grasses IX. Breeding system in *Hordeum glaucum* Steud. Pakistan JBot 17:305-307.

Hatch SL (1980) Chromosome numbers of some grasses from the southwestern United States and Mexico. The Southwestern Naturalist 25: 278-280.

Sabine SJ, Blattner PR (2006) A Chloroplast Genealogy of *Hordeum* (Poaceae): Long-Term Persisting Haplotypes, Incomplete Lineage Sorting, Regional Extinction, and the Consequences for Phylogenetic Inference. Mol Biol Evol 23(8):1602-1612.

Kalendar R, Tanskanen J, Immonen S, Nevo E, Schulman AH (2000) Genome evolution of wild barley (Hordeum spontaneum) by BARE-1 retrotransposon dynamics in response to sharp microclimatic divergence. Proc Natl Acad Sci USA 97:6603-6607.

Koba T (1993) Phytotechnology no Kokoromi (19). Agriculture and Horticulture 68:515-523.

Levan A, Predga K, Sandberg A (1964) Nomenclature for centromeric position on chromosomes. Hereditas 52:201-220.

Mobayyen S (1981) Seedlings of Iran and Plora of Vascular Plants. Vol. 1. Tehran University Publication, Tehran, Iran.

Nicoloff H, Anastassova-Kristeva M, Kunzel G, Rieger R (1977) The behaviour of nucleolus organisers in structurally charged karyotypes of Barley. Chromosoma 62:103-109.

Parsa A (1950) Plora de L'lran. Vol. 4. Publications du ministere de Teducation. Mseun d'historic naturelle de Tehran.

Petersen G, Seberg O (1997) Phylogenetic analysis of the Triticeae (Poaceae) based on *rpoA* sequence data. Mol Phylogenet Evol 7:217-30.

Romero-Zarco C (1986) A new method for estimating karyotype asymmetry. Taxon 35:526-530.

Seberg O, Prederiksen S (2001) A phylogenetic analysis of the monogenomic Triticeae (Poaceae) based on morphology. Bot. J. Linn. Soc., 136:75-97.

Semenov VI (1986) Vnutrikhromosomnaja topografija geterokhromatina u zlakovykh. Bjulleten' Glavnogo Botaniceskogo Sada 140:68-73.

Sheidai M, Nasirzadeh A, Kheradnam M (2000) Karyotypic study of *Echinops (Asteraceae)* in Pars Province of Iran. Bot J Lin Soc 134: 453-463.

Sheidai M, Arman M, Zehzad B (2002) Chromosome pairing and B- chromosomes in some *Aegilops* species and populations of Iran. Caryologia 55(3):261-271.

Sheidai M, Noormohamadi Z, Kashani N, Ahmadi M (2003) Cytogenetic study of some rapeseed *(Brassica napus* L.) cultivars and their hybrids. Caryologia 56(4):387-397.

Shewry PR, ed. (1992) Barley: Genetics, Biochemistry, Molecular Biology and Biotechnology. CAB International, Oxford.

Spies JJ, Burger TH, van Wyk SMC (1999) Chromosome studies on African plants. 12. The tribes of subfamily Pooideae. Bothalia 29(2):335-341.

Stebbins GL (1971) Chromosomal Evolution in Higher Plants. Edward Arnold, London.

Stebbins GL (1982) Major trends of evolution in the Poaceae and their possible significance. In Estes JR, Tyrl RJ, Brnnken JN, eds. Grasses and Grasslands: Systematics and Ecology. University of Oklahoma Press, pp. 1-36.

Stebbins GL (1986) Grass Systematics and Evolution: Past, Present and Puture. In Söderström TR, ed. Grass Systematics and Evolution.Smithsonian Institution Press.

Strid A, Pranzen R (1981) In Chromosome number reports LXXIII. Taxon 30:829-842.

Termeh F (1986) New Grasses of Iran and Their Geographical Distribution. Vol. 2. (17). Research Institute of Pest & Diseases of Plants, Tehran, Iran.

Valdés, B, Parra R, Sánchez AM, Díaz MD (1999) Números cromosómicos de plantas de Marruecos, IV. Lagascalia 21(l):235-240.

Watson L, Dallwitz MJ (1992) onwards. The grass genera of the world: descriptions, illustrations, identification, and information retrieval; including synonyms, morphology, anatomy, physiology, phytochemistry, cytology, classification, pathogens, world and local distribution, and references. Version: 28th November 2005. http://delta-intkey.com.

Zhang Zp, Zhang YM, Yuan JZ (1990) A study of the chromosomes of *Triticum aestivum* and *Hordeum distichon* by silver staining. Acta Agrie Univ Henan 24:82-85.

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ARTICLE

Indirect shoot organogenesis and plantlets regeneration from stem of ornamental *Dieffenbachia maculata* cv. Marianna

Mohamed El-Sayed El-Mahrouk[1], Yaser Hassan Dewir[12*]; Nisha Singh[2]

[1]Department of Horticulture, Faculty ofAgriculture, Kafr El-Sheikh University, Kafr El-Sheikh, Egypt, [2]School of Biological and Conservation Sciences, University of Kwa-Zulu-Natal, Westville Campus, Private Bag X54001, Durban, South Africa

ABSTRACT **The present study reports a simple protocol for indirect shoot organogenesis and plantlets regeneration of** *Dieffenbachia maculata* **cv. Marianna using stem segments from in vitro shoot culture. Different concentrations and combinations of indole-3-acetic acid (IAA), 1-naphthalene acetic acid (NAA), 2,4-dichlorophenoxyacetic acid (2, 4-D), 6-benzyladenine (BA), and kinetin (Kin) were used for callus induction and shoot organogenesis. The frequency of callus formation reached 87% and the highest number of shoots per callus was 4.8 for explants cultured on Murashige and Skoog (1962) medium supplemented with 15 mg l'[1] BA + 15 mg l'[1] NAA. Shoots were further grown and rooted on MS hormone free medium. Regenerated plantlets were successfully acclimatized in greenhouse with 100% survival rate.**
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KEY WORDS

Araceae
acclimatization
Dieffenbachia
shoot organogenesis

Dieffenbachia is a genus of tropical plants in the family Araceae noted for their patterned colorful, large leaves. The cells of the Dieffenbachia plant contain needle-shaped calcium oxalate crystals called raphides. If a leaf is chewed, these crystals cause a burning sensation in the mouth and throat, swelling can occur along with a temporary inability to speak and from this effect the plants are commonly called dumb cane. Dieffenbachia is often referred to as the "King of Plants". Members of this genus are extremely common houseplants because of their tolerance to shade. The genus has about 30 species, but the most widely grown cultivars are selections from *D. maculata* and *D. amoena.*

Vegetative propagation of *Dieffenbachia* is difficult, therefore, seedling propagation does not encourage the expansion of the species, moreover, seed production is limited (Henny 1988). In vitro methods of propagation are used for production of ornamental plants to meet the growing demand in both the domestic and the export market. Despite the increasing commercial demand of *Dieffenbachia* plants, only a few protocols for *in vitro* propagation through shoot cultures are published (Knauss 1976; Chase et al. 1981; Voyiatzi and Voyiatzis 1989; Henny et al. 2000; El-Mahrouk et al. 2006).

With the commercial application of *in vitro* propagation of *Dieffenbachia,* new cultivars have been released following selection of somaclonal variants (Chen and Henny 2006). The frequency of somaclonal variants is generally high, and the time required for a new cultivar release can be only 2 to 3 years compared to 7 to 10 years required using traditional breeding methods (Chen et al. 2003; Henny and Chen 2003). Successful use of *in vitro* techniques for producing somaclonal variants depends on the establishment of an efficient method for regenerating a large number of plants indirectly from an intervening callus stage (Arce-Montoya and Rodriguez-Alvarez 2006; Hammerschlag et al. 2006). Recently, indirect shoot organogenesis has been reported in *Dieffenbachia* cv. Camouflage using leaf explants (Shen et al. 2007). The objective of this study was to establish a protocol for inducing indirect shoot organogenesis in *Dieffenbachia maculata* cv. Marianna using stem segments.

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Corresponding author. E-mail: ydewir@hotmail.com

Materials and Methods

Plant material

In vitro plantlets of *Dieffenbachia maculata cv.* Marianna were maintained on MS (Murashige and Skoog 1962) solid medium (30 *g* l^{1} sucrose + 8.0 *g* l^{1} agar with 8.0 mg l^{1} BA) and kept at 25°C and 40 pmol m'^{2} s^{1} photosynthetic photon flux (PPF) (16 h/d) for *4* weeks. Shoots were individually separated and cultured on MS medium without growth regulators. After 3 weeks of culture, those shoots of about 3.0 cm in length were used for the experiments (El-Mahrouk et al. 2006)

Callus induction and shoot organogenesis

Stems obtained from the *in vitro* shoot cultures served as explant sources for callus induction. Stem explants were cut

Table **1.** Effect of IAA, NAA, 2,4-D on callus Induction of *Dieffenbachia maculata* cv. Marianna after 10 weeks in culture.

Auxins concentration (mg l^{-1})		Callus formation %	Callus diameter (mm)	Callus fresh weight (mg)
Auxin free medium		44.3 d[z]	42 bed	98 be
IAA	3	85.3 c	50 ab	105 be
	5	84.2 c	55 a	107 be
	10	85.2 c	54 a	154 a
	15	84.4 c	46 abc	114b
NAA	3	80.8 c	36 cd	80 be
	5	90.2 b	35 d	88 be
	10	93.2 b	44 bed	93 be
	15	91.6 b	54 a	170 a
2,4-D	0.1	98.1 a	40 bed	88 be
	0.5	90.4 b	34d	63 c
	1	84.3 c	43 bed	77 be
	3	0e	0 e	0d

[z] Mean separation within rows by Duncan's multiple range test at 5% level

Table 2. Effect of BA, Kin on callus Induction and shoot organogenesis of *Dieffenbachia maculata* cv. Marianna after 10 weeks in culture.

Cytokinin concentration (mg l^{-1})		Callus fresh weight (mg)	Callus diameter (mm)	Shoot organogenesis %	Number of shoots/ callus
Cytokinin free medium		100 c[z]	42 c	0d	0d
BA	5	1671 ab	67 b	50 b	2.1 b
	10	1323 ab	62 b	35 b	3.0 a
	15	998 b	65 b	72 a	3.1 a
	20	1902 a	89 a	3d	0.3 d
kin	5	1114ab	78 ab	38 b	1.7 c
	10	1174 ab	58 be	38 b	3.5 a
	15	1484 ab	58 be	37 b	3.3 a
	20	834 be	65 b	17 c	0.7 cd

[z] Mean separation within rows by Duncan's multiple range test at 5% level

into 5 mm^2 segments and cultured on MS medium with 3% (w/v) sucrose. A series of experiments were conducted using different auxins at different concentrations as follow: 2,4-Dicholorophenoxyacetic acid (2,4-D) at 0.1, 0.5, 1.0 and 2.0 mg l^{-1}; indole-3-acetic acid (IAA) and 1-naphthalene acetic acid (NAA) at 0, 3, 5, 10 and 15 mg l^{-1}; different cytokinins as benzyladenine (BA) and kinetin at concentrations of 0, 5, 10, 15 and 20 mg l^{-1} and different cytokinin/auxin combinations. Plant growth regulator-free medium served as the control. The pH of the medium was adjusted to 5.8 before autoclaving (at 121 °C and 1.2 kg cm^{-2} pressure for 15 min). Explants were cultured in 100 x 15 mm sterile glass Petri dishes containing 20 ml medium. There were 5 explants per Petri dish and *4* replicates per treatment. Cultures were maintained in dark for 8 weeks and *2* weeks in light at 40 pmol m^{-2} s^{-1} PPF. The

Table 3. Effect of BA, IAA, NAA on callus Induction and shoot organogenesis of *Dieffenbachia maculata* cv. Marianna after 10 weeks in culture.

PGRs concentration (mg l^{-1})	Callus fresh weight (mg)	Callus diameter (mm)	Shoot organogenesis %	Number of shoots/ callus
BA 15	976a[z]	95 a	73 b	2.8 b
BA15 + IAA 10	932 a	87 a	60 b	2.8 b
BA15 + NAA 10	980 a	103 a	87 a	4.8 a
BA 15 + IAA 10+ NAA 15	1048 a	105 a	61 b	2.0 b

[z]Mean separation within rows by Duncan's multiple range test at 5% level

callus fresh weight, callus diameter, number of shoots formed per callus, and callus formation as % of total number of explants, were recorded after 10 weeks of culture.

Development of shoot growth and rooting

Dieffenbachia shoots were separated and cultured on MS medium without hormones for their subsequent growth and rooting. The cultures were kept at 25°C and 40 pmol m^{-2} s^{-1} PPF (16 h/d) for *4* weeks.

Acclimatization

Plantlets at the 3-4 leaf stage were transplanted into culture pots (coffee cups) hlled with sterilized peat moss. The plantlets were grown in growth chamber for 1 week before their transfer to the greenhouse. The environment in the growth chamber was adjusted to a 25 ± 2°C air temperature, 40 - 50% relative humidity and a 100 pmol m^{-2} s^{-1} PPF with a 16 h photoperiod using halide lamps.

Experimental design and data analysis

Experiments were set up in a completely randomized design and repeated twice. Data were subjected to Duncan's multiple range test using SAS program (Version 6.12, SAS Institute Inc., Cary, USA).

Results and Discussion

Stem segments were cultured on MS medium supplemented with various auxins (IAA, NAA and 2,4-D) at different concentrations to determine the optimal auxin concentration for callus induction and growth (Table 1). The percentage of callus formation signihcantly increased at all auxin concentrations tested except that of 2.0 mg l^{-1} 2,4-D in comparison with auxin-free medium (the control). The highest percentage of callus formation was observed on medium containing 0.1 mg l^{-1} 2,4-D. The percentage of callus formation was signihcantly suppressed on medium supplemented with more than 0.1 mg l^{-1} 2,4-D. However, all concentrations of 2,4-D did not promote callus growth in terms of callus diameter and

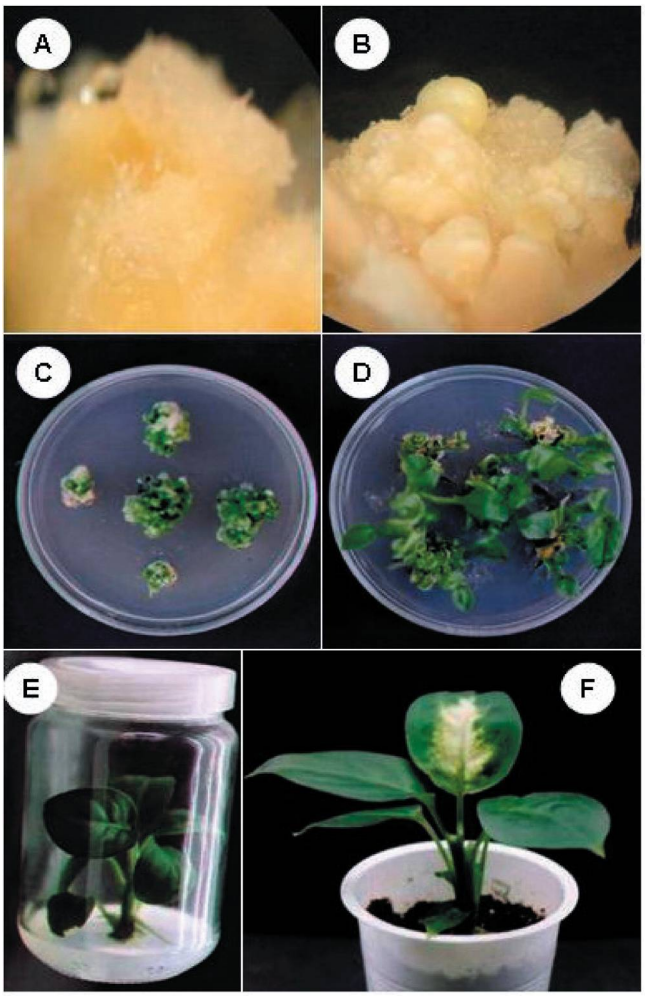

Figure 1. Indirect shoot organogenesis in Dieffenbachia. A, callus formation from stem segments; B, organogenic callus after 6 weeks of culture; C, D, greenish organogenic clusters forming shoots after 10 weeks; E, plantlets rooting in vitro; F, acclimatized plants in the greenhouse.

callus fresh weight. 2,4-D has been proved effective for callus induction in many plant species. In the present work, 2,4-D was not effective for callus growth and was not a prerequisite for callus induction. Similar results were also observed in *Dieffenbachia* cv. Camouflage (Shen et al. 2007). The callus formation observed on auxin-free medium indicates that the explants contained endogenous auxins which were enough to initiate callus. The best response for callus formation %, callus diameter and callus fresh weight was observed on medium containing 15 mg l^{1-} NAA as well as 10 mg l^{1} IAA. The calli were then transferred to MS medium containing different cytokinin types and concentrations and/or MS medium without growth regulators for *4* weeks (data not presented). No features of somatic embryogenesis or shoot organogenesis could be observed.

Callus induction and shoot organogenesis were investigated by testing cytokinins (BA, kin) alone. Shoot organogenesis responses caused by the various concentrations of cytokinins are presented in Table *2.* Proliferation responses were influenced by the cytokinin types and their concentrations. The highest shoot organogenesis percentage (72%) and number of shoots per callus (3.1) were obtained when the culture medium was supplemented with 15 mg l^{1} BA. Also, high shoot number per callus was observed when the culture medium was supplemented with 10 or 15 mg 1^{1} kinetin. However, the shoot organogenesis percentage was low at all the concentrations tested of kinetin. The type and concentrations of cytokinins are the key factors influencing indirect shoot organogenesis in *D. maculata* cv. Marianna. Variation in the activity of different cytokinins may be explained by their different uptake rate reported in different genomes (Blakesley 1991).

When BA was employed with NAA, the number of shoots per callus increased in comparison with treatments with BA alone (Table 3). In general, there were no significant differences in fresh weight and callus diameter at all tested combinations of 15 mg l^{1} BA with NAA or IBA. However, the largest number of shoots, 4.8 per callus, was obtained when the supplied auxin was NAA (10 mg l^{1}) combined with 15 mg 1^{-1}BA. The proportion of auxin-cytokinin is a determinant for meristem formation and the hormone balance that becomes established between growth regulators determines the type of buds induced (George 1993). The inherent endogenous auxin and cytokinin levels must have also played a role in the observed data (Pierik 1987). It is clear from our data that high cytokinin concentration is the main factor influencing indirect shoot organogenesis in *D. maculata* cv. Marianna. Application of NAA in combination with BA increased the number of shoots per callus. These data suggest that one mode of action for auxins could be to down-regulate both local cytokinin synthesis and cytokinin export from medium; this might influence the endogenous cytokinin levels and lead to the activation of buds (Eklof et al. 1997; Sato and Mori 2001).

Nodular calli were observed on the stem segments after 6 weeks of culture on MS medium supplemented with 15 mg l^{1} BA and 10 mg l^{1} NAA (Fig. 1 A, B). Small green meristems were visible on the surface of calli upon their transfer to light conditions. These meristems were later developed into shoot buds upon their subculture to MS medium hormone-free (Fig. 1 C). Shoot clusters with leaves were developed by the end of 10 weeks of culture (Fig. 1 D). The regenerated shoots via indirect shoot organogenesis were separated and cultured for *4* weeks on MS medium hormone-free for their rooting and 100% of plantlets were rooted (Fig. 1 E). *Dieffenbachia* plantlets (3-5 cm) at the 3-4 leaf stage were then grown in growth chamber for 1 week before their transfer to the greenhouse. The plantlets were acclimatized successfully (Fig. 1 F) with 100% survival rate.

In conclusion, the present study reported a simple protocol for in vitro production of *Dieffenbachia* plants via indirect shoot organogenesis. This protocol can be useful in further studies for screening somaclonal variations and developing new cultivars of this important ornamental plant.

References

Arce-Montoya M, Rodriguez-Alvarez M (2006) Micropropagation and field performance of *Yucca valida*. Plant Cell Rep 25:777-783.

Blakesley D (1991) Uptake and metabolism of 6-benzyladenine in shoot proliferation of *Musa* and *Rhododendron*. Plant Cell Tiss Organ Cult 25:69-74.

Chase AR, Zettler FW, Knauss JF (1981) Perfection-137B: a pathogen-free selection of Dieffenbachia *maculata* derived through tissue culture. Circular S-280, Florida Agricultural Experiment Stations, Institute of Food and Agricultural Sciences, University of Florida, pp. 1-7.

Chen J, Henny RJ (2006) Somaclonal variation: an important source for cultivar development of floriculture crops. In Teixeira da Silva JA (ed.) Floriculture, Ornamental and Plant Biotechnology vol II, Global Science Books, London, UK, pp. 244-253.

Chen J, Henny RJ, Chao CT (2003) Somaclonal variation as a source for cultivar development of ornamental aroids. Recent Res Devel Plant Sci 1:31-43.

Eklof S, Astot C, Blackwell J, Moritz T, Olsson O, Sandberg G (1997) Auxin-cytokinin interactions in wild-type and transgenic tobacco. Plant Cell Physiol 38:225-235.

El-Mahrouk ME, El-Tarawy MA, Menesi FA, Metwally AI (2006) Micropropagation of *Dieffenbachia* plants from a single stem-nodes. Internatl J Botany 2(3):324-328.

George EF (1993) Plant Propagation by Tissue Culture. Edington, U.K: Exogenics: p. 574.

Hammerschlag F, Garces S, Koch-Dean M, Stephanie R, Lewers K, Maas J, Smith BJ (2006) In vitro response of strawberry cultivars and regenerants to *Colletotrichum acutatum*. Plant Cell Tiss Org Cult 84:255-261.

Henny RJ (1988) Ornamental aroids: culture and breeding. Hort Rev 10:1-33.

Henny RJ, Chen J (2003) Cultivar development of ornamental foliage plants. Plant Breed Rev 23:245-289.

Henny RJ, Goode L, Ellis W (2000) Micropropagation of *Dieffenbachia*. In Trigiano RN and Gray DJ (ed) Plant Tissue Culture Concepts and Laboratory Exercises, 2nd ed. CRC Press, Boca Raton, London, New York and Washington D.C. p. 97-102.

Knauss JF (1976) A tissue culture method for producing *Dieffenbachia picta* cv. 'Perfection' free of fungi and bacteria. Proc Fla State Hort Soc 89:293-296.

Murashige T, Skoog F (1962) A revised medium for rapid growth and bioassay with tobacco tissue culture. Physiol Plant 15:473-495.

Pierik RLM (1987) In vitro culture of higher plants. Boston: Martinus Nijhoff; pl3.

Sato SS, Mori H (2001) Control in outgrowth and dormancy in axillary buds. Plant Physiol 127:1405-1413.

Shen X-L, Chen J-J, Kane ME (2007) Indirect shoot organogenesis from leaves of *Dieffenbachia* cv. Camouflage. Plant Cell Tiss Org Cult 89:83-90.

Voyiatzi C, Voyiatzis (1989) In vitro shoot proliferation rate of *Dieffenbachia exotica* cultivar 'Marianna' as affected by cytokinins, the number of recultures and the temperature. Sci Hort 40:163-169.

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ARTICLE

Studies on saponins of leaf of *Clerodendron thomsonae* Balfour

Patrick-lwuanyanwu KC[1]*, Sodipo OA[2]

'Toxicological Unit, Department of Biochemistry, University of Port Harcourt, Port Harcourt, Rivers State, Nigeria,[2] Department of Biochemistry, School of Medical Sciences, University of Maiduguri, Maiduguri, Borno State, Nigeria

ABSTRACT In this study, the phytochemical screening of the leaf extracts of *Clerodendron thomsonae* **Balfour were performed to ascertain one of its secondary metabolite constituents, saponin. The results revealed the presence of cardiac glycosides, flavonoids, alkaloids, glycosides and saponin glycosides. However, the plant extract was devoid of tannins, phlobatannins and anthracenes.The presence of saponins was detected by the formation of long-lasting foams and haemolysis of red blood cells. Phosphate-buffered saline (PBS) extract foamed with a height of 9.25 ± 0.4 mm and a foaming time of 20.93 ± 0.56 h while the methanol/phosphate buffered saline (M/PBS) extracts possessed a foam height of 3.75 ± 0.11 mm and disappeared fully after 27.37 ± 0.04 h. Generally, the foaming time was not proportional to the foam height. The melting point of both the aqueous and methanol crude extracts ranged between 283 to 315°C and 125 to 240°C, respectively. PBS and M/PBS extracts possessed haemolytic activities against human erythrocytes (ABO) but at varying degrees. The haemolytic activities for full and partial haemolysis of human red blood cells ranged between 1 to 2[s] and 2[3] to 2", respectively.**
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KEY WORDS

phytochemical screening
secondary metabolites
saponins
foam-forming
full haemolysis
partial haemolysis
haemolytic activity

Saponins are a diverse group of compounds widely distributed in the plant kingdom (Güclü-Ustündag and Mazza 2007). They occur constitutively in a great many plant species, in both wild plants and cultivated crops. In cultivated crops, the triterpenoid saponins are generally predominant, while steroid saponins are common in plants used as herbs or for their health-promoting properties (Fenwick et al. 1991). They are stored in plant cells as inactive precursors but are readily converted into biologically active compounds by plant enzymes (Mert-Türk 2005). They derive their name from their ability to form stable, soaplike foams in aqueous solutions (Shi et al. 2004).

In chemical terms, saponins are glycosylated compounds that are widely distributed in the plant kingdom and can be divided into three groups; a triterpenoid, a steroid, and a steroidal glycoalkaloid. Triterpenoid saponins are found primarily in dicotyledonous plants. They are also found in some monocots, whereas steroid saponins occur mainly in monocots, such as the Lilliaceae, Amaryllidaceae and in certain dicots, such as foxglove (Hostettmann and Marston 1995). Oats (Avena spp.) are unusual because they contain both triterpenoid and steroid saponins (Price et al. 1987). Steroidal glycoalkaloids are found primarily in members of the family Solanaceae, which includes potato and tomato (Osbourn 1996).

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Corresponding author. E-mail: kctrendy@yahoo.co.uk

Saponins are attracting considerable interest as a result of their diverse properties both deleterious and benehcial (Shi et al. 2004). The pharmacological reputation of the saponins used to be like all detergents which on injection, causes lysis of the blood cells, and are highly toxic. This rather negative reputation for saponins has been transformed since the 1960s by closer observation of the action of many herbal remedies, and in particular of the remedy ginseng. It is now accepted that these rather elusive substances are responsible for quite astonishing properties, and in some ways can claim to have challenged the whole edifice of orthodox pharmacology (Herbs2000.com 2006). Because of the presence of both hydrophilic and hydrophobic regions, saponins are excellent emulsihers and foaming agents, and provide functional roles in foods. A high saponin diet can be used in the inhibition of dental caries and platelet aggregation, it is also used in the treatment of hypercalciuria in humans, and as an antidote against lead in epidemiological studies. The ability of saponins to form emulsions in the intestine have lead to the investigation into their role in lowering serum cholesterol in humans (MacDonald et al. 2005). Their toxicity is related to their activity in lowering surface tension (Birk 1969). Saponins have a bitter taste and when ingested orally are practically non-poisonous to warm blooded animals. When injected directly into the blood stream, however, they are dangerous and quickly dissolve red blood cells. Saponins normally break down in the digestive system and must enter the blood

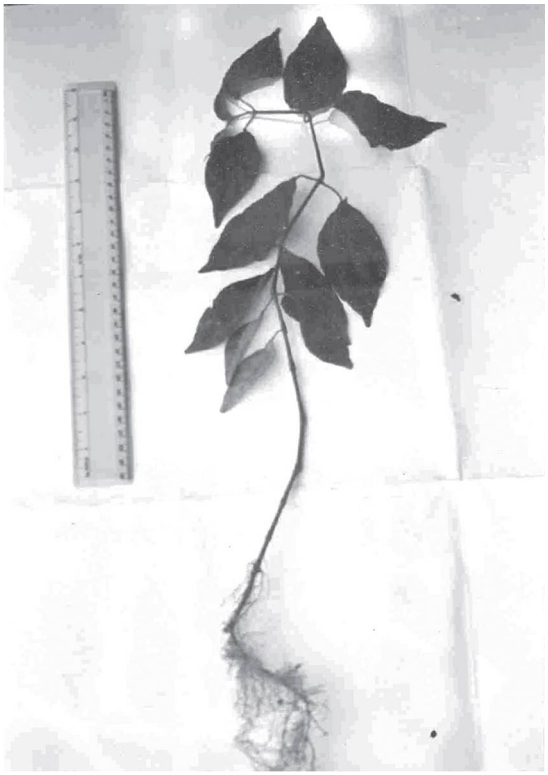

Figure 1. The plant *Clerodendron thomsonae* Balfour.

stream to be toxic (Elpel 2000), but fish assimilate saponin directly into their blood stream via their gills. Fish poisoned by saponins become stupefied and float to the surface where they can easily be collected. Saponins have been shown to inhibit cholinesterase; trypsin and proteinase activities (George 1965). The biological activity of saponin such as anticancer and anti cholesterol activity has led to the emergence of saponins as commercially significant compounds with expanding applications in food, cosmetics, and pharmaceuticals sectors (Gücłü-Ustuňdag and Mazza 2007). They have also been reported to reduce the more harmful LDL-cholesterol selectively in the serum of rats, gerbils and human subjects (Potter 1993; Harris et al. 1997; Matsuura 2001). They are soluble in ethanol, methanol, water or mixture of water with methanol or ethanol (Gennario 1985). Some are exceptions to these characteristics e.g soybean do not form complexes with cholesterol. Ladino clover saponin is not toxic to fish and does not haemolyse red blood cells (Walter et al. 1955). Saponins occur in more than 90 families (Chandel and Rastogi 1980) and at least 500 genera of plants (Basu and Rastogi 1967). A certain plant and even part of the same plant may contain different saponins which can differ in bulbs, blossoms and fruits (Fenwick and Oakenfull 1981). Saponins have been found in legumes, soybean (Fenwick and Oakenful 1981; Shi et al. 2004), sugar beet, spinach asparagus and horse chestnut (Fenwick and Oakenful 1981), Millet *(Pennisetum typhoideum),* bitter Kola *(Garcinia kola),* Cocoyam *(Colocosia esculenta),* guinea com *(sorghum vulgare),* groundnut *(Arachis hypogeae),* sweet potatoes *(Ipomea batata)* and cassava *(Manihot esculenta*; Sodipo and Arinze 1985). The neem, *(Azadirachta indica*\ Sodipo andTizhe 1991).

The biological effects of saponins arise from their steroids and proteins (Dourmashkin et al. 1962). Saponins affect ATPase activity owing probably to a gentle alteration of the erythrocyte membrane. Aescin and whole extracts of horse chestnuts inhibits and reduce the development of atherosclerosis in rabbits and rats (Birk 1969). Ginseng saponin stimulates the renal nuclear and cytoplasmic RNA synthesis and it is therefore suggested that RNA and protein in rat kidneys are stimulated by those saponin which ultimately improve the functions of the kidney (Chandel and Rastogi 1980). By their ability to lower surface tension and possession of emulsifying properties, saponins tend to alter permeability of the cell wall thereby exhibiting a general toxicity on all organised tissues (Basu and Rastogi 1967). Saponins are known to decrease blood lipids, lower cancer risks, and lower blood glucose response (Petit et al. 1993; Kim et al. 1998; Fee et al. 2000; Yoshikawa et al. 2001). Saponins have also been shown to have an inverse relationship with the incidence of renal stones (Shi et al. 2004).

Certain saponin drugs have long been known as fish poisons and for killing fish by artesanal fishermen. They are also considered to be the active components of many traditionally used fish poison (Francis et al. 2001). Saponins have been reported to be highly toxic to fish because of their damaging effect on the respiratory epithelia (Roy et al. 1990; Rooj 1993). Fish have also been shown to exhibit stress reactions to the presence of saponins in water (Francis et al. 2002). Rooj (1993) discovered in the bark and leaves of *Teminalia tomentosa,* a common Indian plant to containjuice saponin consisting of arjunolic acid as its ingredient. This acid is known to be toxic to fish.

The potentials of the class of plant glycoside in the control of schistosomiasis (Bilharziasis) have also been discussed (Lemma 1965; Dorsaz et al. 1988; Kishor and Sati 1990; Sodipo 1998; Apers et al. 2000). The molluscicidal activity of the saponins according to Francis et al. (2002), may be due to their characteristic detergent effect on the soft body membranes of the molluscs.

The plant, *Clerodendron thomsonae* Balfour (Fig. 1) is of the genus Clerodendron consisting of about 400 herbs, vines, shrubs and tress of the tropics and many of which are grown as garden plants. It belongs to the family Verbenaceae, Order Famiales. A world wide but mainly tropical grouping of about 100 genera and over 3,000 species, some of which are important for their flowers. The plant is common in the coastal region of Nigeria. It is mainly used by fishermen in killing fish in a primitive method of fishing. This is achieved

by macerating the leaf of the plant after which it is sprinkled in the river thereby causing marked behavioural change in the hsh. This is characterized by restlessness, fast swimming, occasionaljumping and surfacing responses in the hsh. The hsh die after about 15 minutes of the application of the plant in water. Among the Ikweres in the south - south region of Nigeria, the plant is called "Egwaro".

Materials and Methods

Collection of plant materials

The plant leaf of *Clerodendron thomsonae* Balfour, was collected from two different localities: "Alakahia" and "Aluu" in Rivers State, Nigeria. The plant samples were thoroughly examined to ensure that they were disease-free and then authenticated by the Herbarium section of the Plant Science and Biotechnology Department of the University of Port Harcourt. Nigeria. The identity of the plant was also conhrmed at the forestry Research Institute of Nigeria, Jericho, Nigeria.

Treatment of plant material

After the separation of the plant into various parts, the leaves were subjected to standard heat treatment using the Cosair oven at 80°C for 10 mins prior to rapid drying at 60°C (Joslyn 1970). Further drying was carried out in the sun until it was completely dried. The drying was carried out in order to inactivate the glycosidases present in the leaves which are capable of destroying the saponin activity in the fresh plant tissues. The dried leaves were ground with a clean mortar and pestle and further milled into a hne powder using an electric blender (Moulinex) and made to pass through a 0.25 mm sieves (Ende-cotts (Test sieves) Ltd, England).

Preparation of methanol extract

Aliquots of the ground sample was weighed into whatman paper thimbles (60 mm x 26 mm) and plugged with glass wool. Each aliquot of the sample and glass wool was then covered by a strip of aluminum foil and held in place by a strip of copper wire. The sample (50 g in all) was extracted by glass soxhlet (intermittent with Allihn type of condenser) in 500 ml of methanol (b. pt. 64°C - 65°C) for 24hr.

Preparation of water extract

50g of the plant sample was extracted with 500 ml portion of distilled water using the cold extraction method in a percolator (2L), the tap was plugged with a glass wool and left overnight for extraction with occasional shaking. The extract was then collected by running off the extract through the tap.

Preparation of phosphate-buffered saline (PBS) extract

0.5 g of the plant sample was extracted by macerating with 25 ml PBS using glass mortar and pestle and boiled in boiling water for 30 min. The temperature of the water bath was maintained at 100°C. It was then hltered into 100 ml conical flask, stoppered with cotton wool. The PBS extract was stored at 4°C in a refrigerator until use.

Preparation of methanol/phosphate buffered saline (M/PBS) extract

0.5 g portion of the plant sample was extracted by macerating with 25 ml methanol using a glass mortar and pestle and then transferred into a 100 ml conical flask, stoppered with cotton wool. It was boiled in boiling water for 10 min at 65°C with occasional shaking at 5 min intervals. The temperature of the water bath was maintained at 65°C. After 10 min, it was filtered hot and the methanol filterate evaporated slowly and the residue resuspended in 25 ml PBS. The resulting extract (M/PBS) was then stored at 4°C until use.

Foam forming activities of PBS and M/PBS extracts

The foam forming activity was determined in quadruplicates using a stoppered test tube (17 mm x 175 mm; O'Dell et al. 1959; Oyedapo et al. 1999).

Treatment of erythrocytes

Fresh venous blood was collected from healthy donors into a clean sterile tube containing EDTA (5 mg/8 ml blood) and used within 24 hr. Centrifugation was carried out at 3,000 rpm for 5 min. The supernatant was discarded using Pasteur pipette. Packed cells were washed five (5) times with PBS at 2% (v/v) and stored at 4°C until use (Ralston 1976).

Haemolytic assay

The method used was an adaptation of the Haemagglutination procedure of Gordon and Marquardt (Gordon and Marquardt 1974). Using micro titre plates (Sever 1962) and then modified by Sodipo (Sodipo and Tizhe 1991). Haemolytic assay were conducted by a two - fold serial dilution of the different extracts (PBS and M/PBS) using U-shaped bottom micro titre plates with 2% (v/v) erythrocytes suspension (ABO). PBS (25 pi) was added to each hole 2 to 12, and 50 pi of PBS or M/PBS extract to hole 1, using a dropper. Serial 1:2 dilution was then carried out using micro-dilution by transferring 25 pi from hole 1 into hole 2 through to hole 11 leaving 12 as control and undiluted. The micro diluters were washed with distilled water, rinsed with PBS and dried on a piece of tissue before carrying out fresh titration. The diluter was twiddled and drawn up the side of the hole in hole 1 to dislodge any sample on the outer side of the diluter and then put into hole 2, twiddling and drawing up the side of the hole 1. This procedure was repeated in all the holes up to hole 11, leaving hole 12 in each case undiluted, serving as the control. 25 pi

Table 1. Results of phytochemical screening.

Test	Results
Alkaloids:	
Wagner's reagent	+
Mayer's reagent	+
Tannins	-
Phlobatannins	-
Anthraquinones	-
Combined - Anthraquinones	-
Anthracene	-
Flavonoids:	+
Glycosides	+
Saponin glycosides	+
Lieberman-Burchard	+
Salkowski's	+
Keller-Killian's	-
Foam formation	+
Emulsion	+
$SbCl_3$	-

Table 2. Foam-forming activity of PBS and M/PBS extract of *Clerodendron thomsonae.*

Extract	Foam-forming Activity	
	Foam height (mm)	Foaming time (hr)
PBS	9.25 ± 0.40	20.93 ± 0.56
M/PBS	3.75 ± 0.11	27.37 ± 0.04

Results are the mean value of4 determinations in each case ± SEM

of sample was transferred by micro diluter and mixed in holes with 25 pi PBS, the dilution for example in holes 2 to 6 was 1:2; 1:4; 1:8; 1:16 and 1:32 respectively. The dilution in hole 11 was 1:1024. After dilution, a drop of treated erythrocyte was added into each hole (holes 1 to 12) and incubated at room temperature to give a sedimentation pattern. The sedimentation patterns of the erythrocyte in the undisturbed plates were read after incubating for 2 hr at room temperature (30°C) to determine the titre. A positive pattern indicating full haemolysis (FH) appeared as a circular big spot of red solution surrounded by clear zone (if any), while a negative pattern indicating no haemolysis appeared as a uniform small spot of erythrocytes at the bottom of the well, surrounded by a big concentric clear zone. In some micro titrations where a positive pattern was observed, the clump of erythrocytes was rather large and non-uniformed spot formed. In such cases, a partial haemolysis (PH) was recorded.

Phytochemical screening

The methanolic extract fraction was assayed for the presence of secondary metabolites using standard procedures (Sofowora 1993; Oyedapo et al. 1999). (a) For alkaloids, 0.1 g of the extract was stirred in 10%(v/v) HC1 on a steam bath followed by hltration. The hltrate (1ml) was mixed with a few drops of Mayer's reagent. To another 1ml of the hltrate was added few drops of Wagner's reagent. The mixtures were observed for turbidity or formation of precipitate, (b) Saponins were screened by dissolving 0.1 g of the extract in 2 ml of distilled water, with vigorous shaking until froth appeared. The tubes were warmed for 10 min. in a water bath. The presence or absence of frothing was noted after warming, (c) For tannins, 0.1 g of the extract was taken up in 10 ml distilled water, and hltered. Then, a few drops of ferric chloride reagent were added to the hltrate. The mixture was observed for the formation of blue, blue-black, green or green-black colouration or precipitate, (d) Tests for havonoids involved (i) suspending 0.1 g of the extract in 5ml ethanol, followed by shaking and hltering. To 1ml of the hltrate was added a few drops of 0.5N alcoholic KOH .The mixture was observed for yellowish suspension or precipitate, (ii) (0.1 g) of the extract was suspended in 5 ml of ethylacetate, shaking vigorously and hltered. To lml of the hltrate was added few drops of dilute ammonia solution. The alkaline layer was observed for turning light or deep brown, (e) Cardiac glycosides were screened by dissolving (0.1 g) of the extract in 5 ml chloroform followed by hltration. Concentrated sulphuric acid was carefully layered at the bottom of the tube without disturbing the solution. It was observed for the formation of a sharp brown ring at the chloroform/sulphuric acid interface

Determination of melting point

A few milligram of the sample was forced down through the open end of a clean capillary tube towards the sealed end. The packed tube was then inserted into the heating bore of a melting point apparatus. Having regulated the temperature rise to be gradual, the temperature switch was switched on. The sample went through various stages of physical changes such as:

First change of sign (darkening)
First liquid formation
Meniscus formation and hnally
Complete liquid formation.

All these changes were viewed through a magnifying glass. The temperature range over which this physical changes occurred was determined and recorded as the melting point.

Results

Phytochemical screening of the leaf of *Clerodendron thomsonae* Balfour showed the presence of secondary metabolites (Table 1). However, it was devoid of tannins, Phlobatannins, Anthraquinones, combined- anthraqunones and Anthracene (Table 1).

Foam- forming activity carried out revealed that the PBS extract had a higher foam height when compared with the

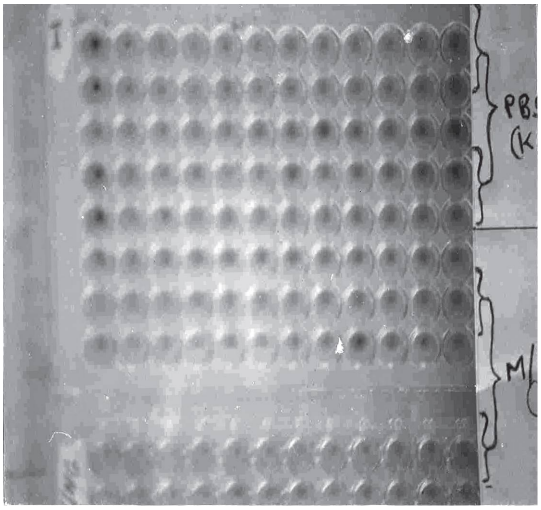

Figure 2. The Haemolytic pattern of PBS and M/PBS of *Clerodendron thomsonae*_**Balfour.**

Table 3. Determination of haemolytic activity of crude saponin extracts (PBS and M/PBS).

Extract	Pattern of Haemolysis	Titre		
		A	B	O
PBS	FH	2^5	2^7	2^0
	PH	2^9	2^9	2io
M/PBS	FH	2^4	2^6	2^0
	PH	2^9	2io	2^3

Titre is defined as the reciprocal of greatest dilution at which haemolysis occurred.
All values are mean of duplicate determination.
FH - Full haemolysis, PH - Partial haemolysis.

Table 4. Results of determination of melting point.

Extract	°C
Aqueous	288-315
Methanol	125-240

M/PBS extract. However, the M/PBS extract showed a longer foaming time when compared to the PBS extract (Table 2). The results of the haemolytic assay carried out on both the PBS and M/PBS extract of the leaf of the plant *Clerodendron thomsonae* Balfour, were found to posses haemolytic activity with different human erythrocytes (Table 3). Partial and Full haemolysis for both PBS and M/PBS extracts were observed with human erythrocytes (Fig. 2). The result obtained from haemolytic activity of the PBS extract revealed that blood group O recorded the least titre while blood group A recorded the highest titre with respect to full haemolysis (FH). However, in the partial haemolysis result of PBS extract, blood group O recorded the highest titre, whereas the same but lower titre was recorded for both blood group A and B (Table 3). The M/PBS extract results obtained for full haemolysis (FH) showed that blood group B recorded the highest titre while blood group O recorded the lowest titre. Blood group B also recorded the highest titre with respect to partial haemolysis (PH) while blood group O recorded the lowest titre (Table 3).

The results of the determination of melting point (m. pt) showed the melting point range of both methanol and aqueous extract of the leaf of *Clerodendron thomsonae* Balfour. The result revealed that the aqueous extract had a higher melting point range than the methanol extract (Table 4).

Discussion

The phytochemical screening of the leaf extract of *Clerodendron thomsonae* Balfour, was studied for the presence of various secondary metabolite among which is saponin. The result obtained from this research showed the presence of saponin in the leaf. Further confirmatory test for saponins were achieved in view of the positive responses to Fehlings solution. The sugar portion were most probable those of saponins, since it is known that a particular saponin can contain as many as twelve different sugar or saccharic acid units (Kitagawa et al. 1975). These sugars are regarded as united and in a straight chain with the terminal unit attached to a OH guinyl of the sapogenin through glycosidic linkage (Birk 1969).

The result of the study showed that the PBS and M/PBS extract of the leaf of *Clerodendron thomsonae* Balfour, not only foamed copiously but also formed foams that lasted for a considerable length of time. The PBS extracts had a higher foam height of 9.25 ± 0.4 mm while that of the M/PBS extract was 3.75 ±0.11 mm. The foaming time for PBS extract was 20.93 ± 056 while M/PBS extract had a foaming time of 27.37 ± 0.04 hr. This result is in agreement with Sodipo and Mohammed (1990), Sodipo and Tizhe (1991) that the foaming time is not always proportional to the foaming height.

Furthermore, the PBS and the M/PBS extract of the leaf were found to posses haemolytic activity using different human erythrocytes. Generally, the results obtained with blood group O showed a very mild activity. However, results of the haemolytic activity observed with both PBS and M/PBS extract could be attributed to the saponin content of the plant extract (James 1964; Newbeme 1980; Khalil andEl-Adawy 1994). This toxicologically interesting property of many saponins to bring about haemolysis *i.e.* the release of haemoglobin from erythrocytes is as a result of change in membrane permeability. This is considered to be influenced by the affinity of the aglycone to cholesterol in cell membranes (Glauert 1962; Frobbe 1992). The ability of the plant extracts to lyse red blood cells at varying degrees could be attributed to the presence of different types of saponins as was reported in

the findings of Khalil and El-Adawy (1994) and Oda et al. (2000) where different saponins showed different levels of haemolytic activities. This observation can also be correlated to the findings of Fenwick and Oakenfull (1981) that a certain plant and even part of the same plant may contain different saponins which can differ in biological features. However, this haemolytic activity only takes place on parenterial administration since by mouth there is usually only limited absorption. Walter et al. (1955) reported that Ladino Clover saponin is not toxic to fish and does not haemolyse red blood cells. This could have been the reason for the titre result obtained in full haemolysis of both PB S and M/PB S extract of blood group O where a titre of 2° (no haemolysis) was recorded.

The results of determination of melting point (m.pt) revealed that the aqueous extract had a higher melting point than the methanol extract. The observation simply showed that results were obtained as mixtures of various compounds hence the melting point range between 288 to 315°C and 125 to 240°C for both aqueous and methanol extract respectively. This observation is in agreement with the findings of Shi et al. (2004) that saponins constitute a complex and chemically diverse group of compounds. Melting points normally indicate the extent of purity of substances. A pure sample usually has a sharp and definite melting point while an impure or mixed sample will have a melting point range due to the differences in melting points of the substances present in the mixture.

The ability of the leaf of *Clerodendron thomsonae* to kill fish on application in water in a similar manner to *Teminalia tomentosa* could also be attributed to the presence of arjunolic acid in the saponins of leaf of *Clerodendron thomsonae* Balfour.

The presence of saponin earlier detected by the ability of the aqueous extract to form a stable froth when vigorously shaken and also the ability to haemolyse red blood cells was eventually confirmed in view of the positive responses to Fehling's solution. Again, the steroidal portions were detected by the Lieberman-Burchard's and Salkowski's reagents. The haemolysin responsible for the haemolytic activity of the plant extract could be attributed to the presence of saponin. However, for a more conclusive and unambiguous identification of saponins, purification and structural elucidation exercises should be carried out. These should form major components of future research efforts.

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References

Apers S, Baronikova S, Sindambiwe JB, Witvrouw M, De Clercq E, Vanden Berghe D, Van Marck E, Vlietinck A, Pieters L (2000) Antiviral, haemolytic and molluscicidal activities of triterpnoid saponins from *Maesa lanceolata:* establishment of structure-activity relationships. Planta Medica 67:528-532.

Basu N, Rastogi RP (1967) Triterpenoid Saponins and Sapogenins Phytochemistry 6:1249-1270.

Birk Y (1969) Saponins. In Toxic constituents of plant food stuffs, (ed. Liener, I. E.) Academic Press Inc. NewYorkpp. 169-210.

Chandel RS, Rastogi RP (1980) Triterpenoid Saponins and Sapogenins, 1973-1978. Phytochemistry 19:1889-1908.

Dorsaz AC, Hostettmann M, Hostettmann K (1988) Molluscicidal saponins from *Sesbania sesban*. Planta Medica 54:225-227.

Dourmashkin RR, Dougherty RM, Harris RJ (1962) Electron microscopic observation on Rous sarcoma virus and cell membranes. Nature 194:1116-1117.

Elpel TJ (2000) Botany in a Day: Thomas J Elpel's field Guide to plant families, 4th ed. HOPs Press, Pony Montana, USA.

Fenwick DE, Oakenful D (1981) Saponin content of soybean and some commercial saponin products. J Sci Food Agric 32:273-278.

Fenwick GR, Price KR, Tsukamoto C, Okubo K (1991) Saponins. In Saponin in Toxic Substances in Crop Plants, [FJP D'Mello, CM Duffus, editors]. Cambridge: The Royal Society of Chemistry.

Francis G, Kerem Z, Makkar HPS, Becker K (2002) The biological action of saponins in animal system: a review. Br J Nutr 88:587-605.

Francis G, Makkar HPS, Becker K (2001) Antinutritional factors present in plant-derived alternate fish feed ingredients and their effects in fish. Aquaculture 199:197-227.

Frobbe P (1992) A clour atlas of poisonous plants. Wolfe publishing ISBN 0723408394, pp. 26-28.

Gennario AR (1985) Remington's pharmaceutical Sciences (100yrs). 17th edn, Mack publishing Co., Pennsylvania USAp.403.

George AJ (1965) Fegal status and toxicity of saponin. Food Cosmet Toxicol 3:85-91.

Glauert AM, Dingle JT, Fucy JA (1962) Action of saponin on biological cell membranes. Nature 196:952-955.

Gordon JA, Marquardt MD (1974) Glutaraldehyde fixation and mechanism of erythrocyte agglutination by concanavalin A and soybean agglutinin. Biochem Biophys Acta 332:136-144.

Güclü-Ustündag O and Mazza, G (2007). Saponins: properties, applications and processing. Crit Rev Food Sci Nutr 47(3):231-258.

Harris WS, Dujovne CA, Windsor SL, Gerrond LLC, Newton FA, Gelfand (1997) Inhibiting cholesterol absorption with CP-88,818 (beta-tigogenin cellobioside; tiqueside): studies in normal and hyperlipidemic subjects. J Cardiovasc Pharmacol 30:55-60.

Herbs2000.com (2006) Saponins @ www.herbs2000.com © 2002-2006.

Hostettmann KA, Marston A (1995) Saponins. Chemistry and pharmacology of natural products. Cambridge University Press, Cambridge, United Kingdom.

James WO (1964) (ed). Plant Biochemistry, Botanical Monographs Vol.3. Blackwell Scientific Publications, Oxford, p. 410.

Joslyn MM (1970) Methods in food analysis, Academic press Inc. New Yorkpp. 50-53.

Khalil AH, El-Adawy TA (1994) Isolation, identification and toxicity of saponin from different legumes. Food Chem 50:197-201.

Kim SJ, KmYY, Ko KH, Hong EK, Han YB, Kang BH, Kim H (1998) Butanol extract of 1:1 mixture of Phellodendrom cortex and Aralia cortex stimulates P13-linase and ERK2 with increase of glycogen levels in HepG2 cells. Phytother Res 12:255-260.

Kishor N, Sati OP (1990) A new molluscicidal spirotanol glycoside of *Yucca alufólia.* J Nat Prod 53:1557-1559.

Kitagawa I, Imada A, Yoshioka I (1975) Saponin and sapogenol. XIIM1 Saponin Aa and Ml-saponin B, two major bidesmosides from the seed of karnel of *Madhuca lingofolia* (F) Macbride. Chem Pharm Bull 23:2268-2278.

Lee KT, Sohn IC, Kim DH, Choi JW, Kwon SH (2000) Hypoglycaemic and hypolipidemic effects of tetrogenin and kaika saponin III in the streptozotocin-induced diabetic rat and their antioxidant activity in vitro. Arch

Pharm Res 23:461-466.

Lemma A (1965) A preliminary report on the molluscicidal property of endod *(Phytolacca dodecandra)*. Ethiopian Medical Journal 3:187

MacDonald RS, Guo J, Copeland J, Browning Jr JD, Sleper D, George ER, Berhow MA (2005) Environmental Influences on Isoflavones and Saponins in Soybeans and their role in Colon Cancer. J Nutr 135(5):1239-1242.

Matsuura M (2001) Saponins in garlic as modifiers of the risk of cardiovascular disease. Journal of Nutrition 131:1000S-1005S.

Mert-Türk F (2005) Saponins versus plant fungal pathogens. Journal of Cell and Molecular Biology 5:13-17.

Newberne PM (1980) Naturally occurring food borne toxicants. In Modern nutrition in health and disease. Good Hart S and Shills ME (eds). Lea and Febiger, Philadelphia, pp.463-496.

O'Dell BL, Reagan WO, Beach JJ (1959) Toxic principle in red cover. Missoun Univ Agroc Expt Sta Res Bull702:12-13.

Oda K, Matsuda H, Murakami T, Katayama S, Ohgitani T, Yoshikawa M (2000) Adjuvant and haemolytic activities of 47 saponins derived from medicinal and food plants. Biol Chem 381:67 -74.

Osbourn, A.E (1996) Preformed antimicrobial compounds and plant defense against fungal attack. Plant Cell 8:1821-1831.

Oyedapo OO, Sab FC, Olagunju JA (1999) Bioactivity of fresh leaves of *Lantana catnara.* Biomed Letters 59:175-183.

Petit PR, Sauvaire Y, Ponsin G, Manteghetti M, Fave A, Ribes G (1993) Effects of a fenugreek seed extract on feeding behaviour in the rat: metabolic endocrine correlates. Pharmacology Biochemistry and Behaviour 45:518-524.

Potter SM, Jimenez-Flores R, Pollack J, LoneTA, Berber-Jimenez MD (1993) Protein saponin interaction and its influence on blood lipids. J Agric Food Chem 41:1287-121.

Price KR, Johnson IT, Fenwick GR (1987) The chemistry and biological significance of saponins in food and feeding stuffs. Crit Rev Food Sci Nutr 26:27-133.

Ralston GR (1976) Physico-Chemical characterization of spectrum tetramer from bovine erythrocyte membranes Biochem Biophys Acta, 455:163-172.

Rooj NC(1993) Effects of piscicidal plant Terminalia tomentosa juice on the behaviour and gill histopathology of a hill-stream loach. J Ecotoxicol Environ Monit l(3):35-40.

Roy PK, Munshi JD, Dutta HM (1990) Effect of saponin extracts on morphohistory and respiratory physiology of an air breathing fish, Heteropneustes fossilis (Bloch). Journal of Freshwater Biology2:135-145.

Sever JC (1962) Application of microtechnique to viral serological investigation. J Immunol 88:320-329.

Shi J, Arunasalam K, Yeung D, Kakuda Y, Mittal G, Jiang Y (2004) Saponins from edible legumes: chemistry, processing, and health benefits. J Med Food 7(l):67-78.

Sodipo OA, Arinze HO (1985) Saponin content of some Nigerian Foods. J Sci Food Agric 36:407-408.

Sodipo OA (1998) Plant saponins as molluscicides in control of schistosomiasis. Abstract. A publication of the Nigerian Journal of Parasitology. 19:45.

Sodipo OA, Mohammed SL (1990) Foam-forming and haemolytic activities of the extract of Baobab tree, *Adasonia digital* Nigerian Journal of Basic and Applied Science, 4(182):41-52.

Sodipo OA, Tizhe FS (1991) A preliminary study of the saponin content of Neem tree Azadirachta indica A. Juss Annal of Borno 819:142-149.

Sofowora EA (1993) Phytochemical Assays. In Medicinal Plants and Traditional medicine in Africa. 3rd Edition, Spectrum Books Limited Nigeria, pp. 150-153.

Walter ED, Bickoff EM, Thomson CR, Robinson CH, Djerassi C (1955) Saponin from Ladino Clover *(Trifolium reopens).* J Am Chem Soc 77:4936-4937.

Yoshikawa M, Murakami T, Kshi A, Kageura T, Matsuda H (2001) Medicinal flowers. III. Marigold (1): hypoglycaemic, gastric emptying inhibitory, and gastroprotective principles and new oleanane-type triterpene oligoglycosides, calendasaponins A, B,C and D, from Egyptian Calendular officinalis. Chem Pharm Bull 49:863-870.

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ARTICLE

The leaf architecture and its taxonomic significance in Capparaceae from Egypt

Monier M Abd El-Ghani[1]*, Wafaa Kamel[2], Mona El-Bous[2]

[1]The Herbarium, Faculty of Science, Cairo University, Giza, Egypt, [2]Botany Department, Faculty of Science, Suez Canal University, Ismailia, Egypt

ABSTRACT The paper deals with the leaf architecture of **19** species belonging to **7** genera ***(Capparis, Cadaba, Boscia, Maerua, Dipterygium, Cleome*** and ***Gynandropsis)*** of the family Capparaceae ***sensu lato*** (including Cleomaceae) from Egypt. A comprehensive description of leaf architecture for the studied taxa was provided, including venation pattern, areolation, and marginal ultimate venation. The venation pattern showed mostly pinnate brochidodromous or craspidododromous. Aeroles may be well or poorly developed. Taxonomically significant leaf features of the examined taxa showed great diversity in leaf or leaflet form, leaf surface, leaf base, leaf persistence, main venation pattern, secondary vein angle, inter secondary veins, number of veins on either side of midrib, free ending ultimate veins and marginal ultimate venation. A new free ending ultimate venation (F.E.V.S) branched with swollen ends was reported. On the basis of leaf architecture, we advocate the recognition of two separate families Capparaceae ***sensu lato*** and Cleomaceae. Multivariate analysis was carried out with the aim of solving some of the taxonomic problems existing in the family using **21** characters including **74** character states. Based on the comparison of leaf architecture, we supported the taxonomic treatment of the family Capparaceae. We supported retaining ***Gynandropsis gynandra*** as ***Cleome gynandra*** of the family Capparaceae, as it clearly nested within ***Cleome.*** Leaf architecture helped to distinguish all the species investigated and accordingly a key was provided for this purpose.
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KEY WORDS

Dicotyledons
Capparaceae
Cleomaceae
leaf architecture
leaf venation
flora of Egypt
taxonomy

Family Capparaceae *sensu lato* is a fairly large (45 genera and 675 species), mainly subtropical being most conspicuous in tropical seasonally dry habitats with diversity in floral structure (Mabberley 1987). Except in some species of *Capparis,* it has a great constancy in the number and position of sepals and carpels (Pax and Hoffmann 1936; Jacobs 1965). It also shows great diversity in the morphology and number of petals and stamens (Endress 1992). Many genera that were considered in Capparaceae by Pax and Hoffmann (1936) have been elevated to familial level or included in unrelated families. The two major subfamilies of Capparaceae: Cleomoideae (about 8 genera and 275 species) and Capparoideae (about 25 genera and 440 species) are quite distinct, and have been elevated to familial status by some authors (e.g., Airy Shaw 1965; Hutchinson 1967). In both subfamilies the type genus is by far the largest and houses the majority of the species: *Cleome* (200 species) for the former and *Capparis* (150-200 species) for the latter. However, Pax and Hoffman (1936) described the most comprehensive taxonomic treatment of *Capparis* to date in which they recognized 45 genera (20 monotypic) to be included in eight subfamilies.

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Corresponding author. E-mail: elghani@yahoo.com

Capparaceae are represented in the Egyptian flora by 7 genera, 21 species and *4* varieties of wide ecological and geographical range of distribution (Boulos 1999). They vary considerably in their growth forms from small trees *(e.g.Boscia)* or shrubs (e.g. *Capparis)* to annual (e.g. *Gynandropsis gynandra)* or perennial herbs (e.g. *Cleome).* Therefore, their vegetative characters range from woody perennials to annual herbs. The Egyptian taxa of Capparaceae belong to the xerophytic communities (Zahran and Willis 1992; Abd El-Ghani and Marei 2006), except for *Gynandropsis gynandra* that is common among the weed flora of the arable fields (Boulos and El-Hadidi 1984). The taxonomic treatment of the family in Egypt focused mainly on seed morphology (Al-Gohary 1997), leaf anatomy (Al-Gohary 1982) and pollen morphology (Khafagi and Al-Gohary 1998). The systematic revision of the native species of Capparaceae (excluding *Cleome)* revealed the uncertain occurrence of *Boscia angustifolia,* while *Capparis spinosa* is represented by 3 varieties viz.: *spinosa, inermis* and *deserti* (El-Karemy 2001). Separation from Cleomaceae may be unsustainable, since difficulties are encountered in assigning the genera. Precise comparative data on gynoecium and fruit structure are elusive or non-existent. Actually, the taxonomic affinities between Capparaceae and Cleomaceae are still of debate. Täckholm (1974) distinguished between the two families according to gland struc-

Table **1**. List ofthe studied Egyptian taxa arranged into subfamilies and tribes according to Pax and Hoffman (1936).

No	Taxon	Subfamily	Tribe	Numberofexamined individuals
1	Capparis decidua Edgew.	Capparoideae	Capparideae	20
2	Capparis sinaica Veill.	Capparoideae	Capparideae	30
3	Capparis spinosa L. var. spinosa	Capparoideae	Capparideae	30
4	Capparis spinosa L. var. canescens Coss.	Capparoideae	Capparideae	15
5	Capparis spinosa L. var. inermis Turra	Capparoideae	Capparideae	20
6	Capparis spinosa L. var. deserti Zohari	Capparoideae	Capparideae	25
7	Cadaba glandulosa Forssk.	Capparoideae	Capparideae	5
8	Cadaba farinosa Forssk.	Capparoideae	Capparideae	7
9	Cadaba rotundifolia Fords	Capparoideae	Capparideae	10
10	Boscia senegalensis Poir.	Capparoideae	Capparideae	8
11	Boscia angustifolia A. Rich.	Capparoideae	Capparideae	7
12	Maerua oblongifolia (Forssk.) A. Rich.	Capparoideae	Maerueae	5
13	Maerua crassifolia Forssk.	Capparoideae	Maerueae	5
14	Dipterygium glaucum Decne.	Dipterygioideae		10
15	Cleome droserifolia (Forssk.) Delile	Cleomoideae		30
16	Cleome chrysantha Decne	Cleomoideae		12
17	Cleome arabica L.	Cleomoideae		15
18	Cleome brachycarpa DC.	Cleomoideae		5
19	Cleome hanburyana Penz.	Cleomoideae		10
20	Cleome paradoxa R. Br. ex DC.	Cleomoideae		10
21	Cleome amblyocarpa Barratte& Murb.	Cleomoideae		40
22	Gynandropsis gynandra (L.) Briq.	Cleomoideae		20

ture, fruit type, and development of a gynophore, whereas Zohary (1966) included the intriguing genus *Cleome* in the subfamily Cleomoideae of Capparaceae. The Capparaceae in Boulos (1999), however, included both Cleomaceae and Capparaceae. On the species level, Tackholm (1974) recognized 8 species of *Capparis,* whereas Boulos (1999) classihed the genus as 3 species and 4 varieties.

Although flower and fruit characters have proved very useful in identification and delimitation of the genera and species, there are situations in which these organs are not available for study as in Capparaceae. The study of the reproductive characters of this group is problematic for different reasons, amongst others; the difficulty of preserving the flowers in some genera as in *Capparis* (Hedge and Lamond 1970), the striking variability in their size and shape at the individual level species (Mabberley 1987), and many long-lived tropical plant flowers are infrequent and irregular (LAWG 1999). So, there is a great need to identify and classify plants using vegetative characters.

Ettingshausen (1861) made the first comprehensive effort to systematize the description of the vegetative leaf architecture with his classification of venation patterns. Leaf architectural characters have proved valuable taxonomic and systematic data both in fossil and living plants (Hickey 1973; Dilcher 1974; Hickey and Wolfe 1975). Leaf architecture and venation pattern studied in different families of dicotyledons; amongst others, Compositae (Banerjee and Deshpande 1973), Solanaceae (Inamdar and Murthy 1978), Bignoniaceae (Jain 1978), Hamamelidaceae *sensu lato* (Li and Hickey 1988), Leguminosae (Sun et al. 1991), Amaranthaceae (Shanmuka et al. 1994), Ulmaceae (Wang et al. 2001), Lagaceae (Luo and Zhou 2002), and in some monocots (Inamdar et al. 1983). The present work was undertaken to give comprehensive account of the venation pattern and leaf architecture in 7 genera and 19 species of the Capparaceae (including Cleomaceae) as no report exists on the subject. It is a contribution towards better understanding the systematic treatment of the Egyptian Capparaceae verifying the role of leaf architecture, assessing the range of variation among species by applying multivariate analysis.

Materials and Methods

During the growing seasons in 2005-2006, fresh material of 7 genera *(Capparis, Cadaba, Boscia, Maerua, Dipterygium, Cleome* and *Gynandropsis)* and 19 species were collected from their natural habitats and field observations were made from several localities of the Mediterranean region, and in the western Desert, Eastern Desert, Mountains of Sinai and Elba (Fig. 1). In addition, leaves were obtained from herbarium specimens in Cairo University (CAI), Ministry of Agriculture (CAIM) and National Research Centre (CAIRC). In order to broadly sample the variation, the studied taxa were represented by a number of collections (herbarium specimens or fresh material or both) from different localities in Egypt (Table 1). Plant identifications were according to Zohary (1966), Tackholm (1974), Thulin (1993) and Boulos (1999).

Mature leaves were cleared following Thakur (1988), but with modified procedure to suit investigation. Accord-

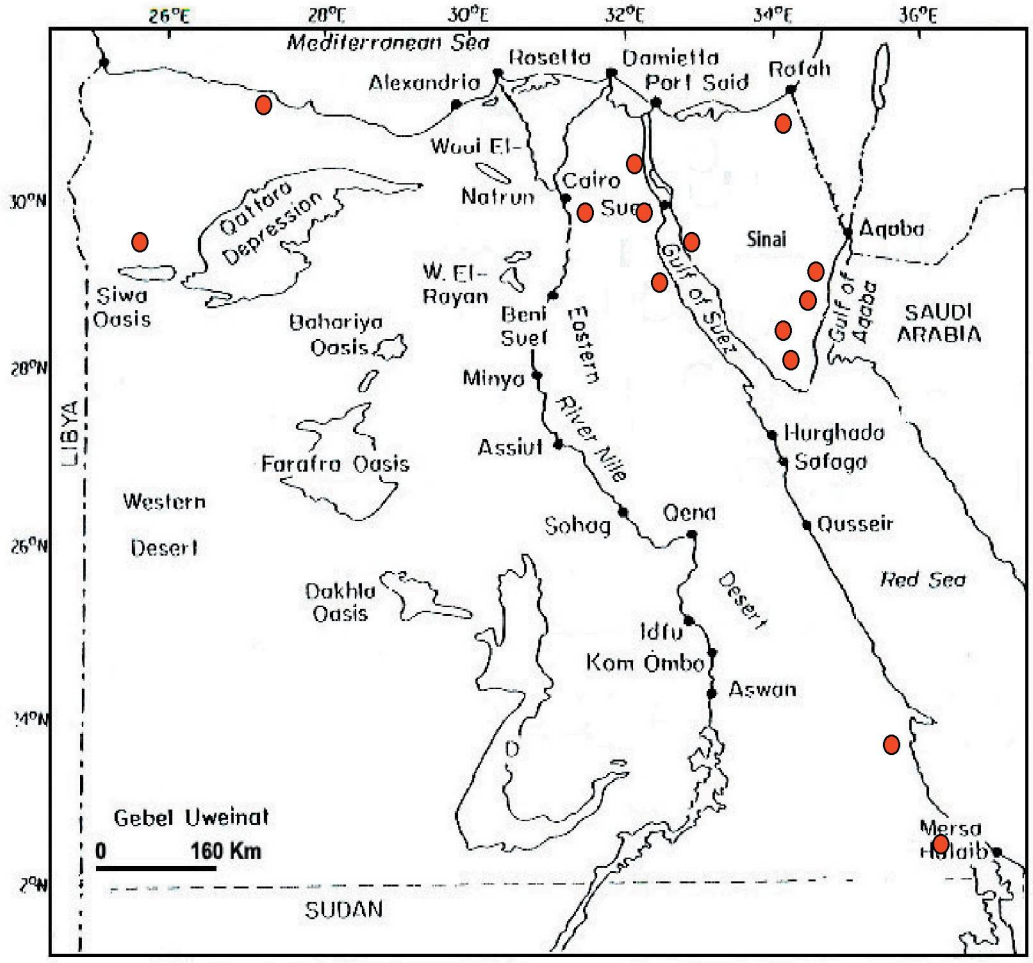

Figure 1. Location map showing the collecting sites.

ingly, the mature leaves were cleared by keeping them in 5% NaOH solution at 25°C for two or three days, rinsed in water and transferred to acetic acid, hydrogen peroxide and lactophenol (Subrahmanyan 1999) in 1:1:1 ratio for three or four days. The cleared leaves were stained with 1% safranin, and mounted on slides with glycerine (Plates 1-3). A total of 21 characters were measured in each studied specimen, comprising 3 quantitative and 18 qualitative characters. Seven of the qualitative characters were scored as binary and the rest were scored as multistate characters (Table 2). The measurements for all specimens of a taxon were averaged into one score for each of the characters. Scores for quantitative characters were averages of measurements of at least 20 specimens (where possible). Because herbarium specimens cannot be considered to be a random sample of the species, we followed Wieringa (1999) by calculating the mean of the minimum and maximum measurement. When some of the characters for a certain species were lacked, these omissions were coded as missing data (-999). The complete data matrix is available upon request from the hrst author. Leaf architectural terminology was largely from Hickey (1973, 1977 and 1991), Levin (1986) and Leaf Architecture Working Group (LAWG 1999).

To avoid the effects of different scales of measurement for different characters, the values for each character were standardized prior to analysis using the default option in SYSTAT version 5.02 for Windows software (SYSTAT Inc, USA). Two types of analyses were performed with Community Analysis Package (CAP version 1.2, Pisces Conservation Ltd, UK). Firstly, we performed three different procedures of agglomerative cluster analysis (complete linkage, average linkage and minimum variance) using Euclidean distance to a data matrix of 22 taxa and 21 characters. Secondly, we performed a principal components analysis (PCA).

Results

Leaf was persistent in most of the studied species, but it was deciduous in only two species; *Capparis decidua* and *Dip-*

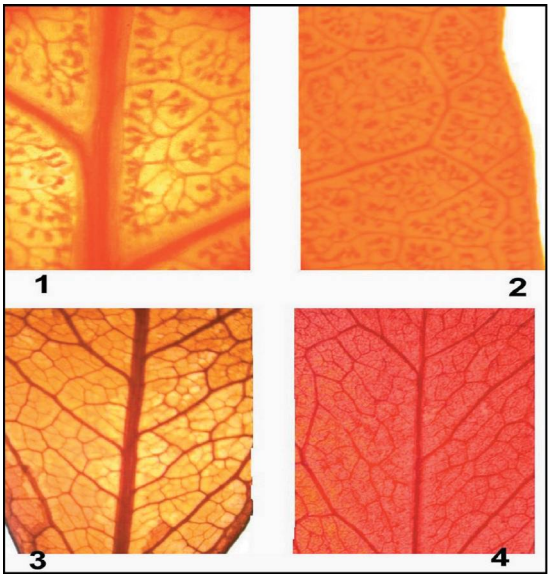

Plate (1). Figure 1: *Capparis spinosaMar. inermis(xAO),* pinnate venation pattern, F.E.V.Sfree ending ultimate veins three branched with swollen dots; Figure 2: *Capparis sinaica* (x40) brochidodromous, incomplete margin; Figure 3: *Boscia senegalensis(x*40), absence of F.E.V.S, random reticulate of third and fourth vein category, prismatic attached vein angle; Figure 4: *Maerua crassifolia* (x40), five or more sided aeroles, tapering branched F.E.V.S.

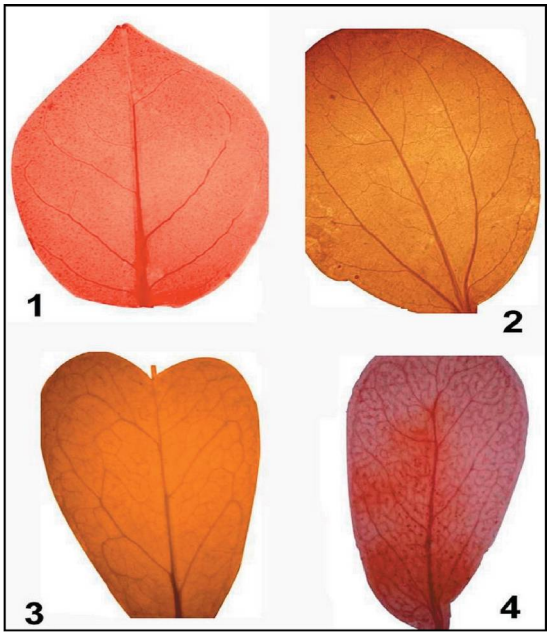

Plate (2). Figure 1: *Capparis spinosa* (x 40), brochidodromous, 4 veins on either side of midrib; Figure 2: *Cleome arabica,* actinododromous, one vein on either side of midrib, cladododromous; Figure 3: *Maerua crassifolia* (x25), secondary vein spacing increasing toward base, secondary vein angle decreasing toward base, marginal ultimate venation of incomplete loops. Figure 4: *Dipterygium glaucum* (x25), cladododromous, agrophic, excurrentvein branched.

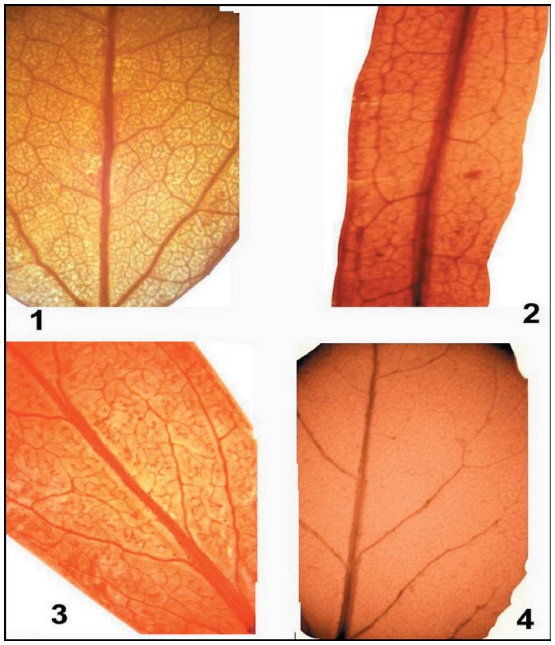

Plate (3). Figure 1: *Boscia angistifolia* (x40), one acute pair secondaries, random reticulate fourth vein category, no F.E.V.S.; Figure 2: *Capparis decidua* (x25), marginal arcuate venations looped arcuate, perpendicular third vein angle to the primary; Figure 3: *Dipterygium glaucum* (x 40) poorly developed aerolation. Figure 4: *Gynandropsis gynandra,* cladododromous, excurrent vein branched.

terygium glaucum. Taxonomically significant leaf features of the examined taxa showed great diversity in leaf or leaflet form, leaf surface, leaf base, leaf persistence, main venation pattern, secondary vein angle, inter-secondary veins, number of veins on either side of midrib, free ending ultimate veins and marginal ultimate venation. As in the case with other taxonomic characters, great care must be taken when using leaf venation.

A survey of venation of Egyptian Capparaceae (Plates 1-3) showed that venation pattern is mostly pinnate, but it was actinododromous in *Cleome arabica* and *C. droserifolia.* Secondary vein category was brochidodromous in the majority of the studied taxa, while it was cladododromous in *Cleome arabica, Dipterygium glaucum* and *Gynandropsis gynandra',* craspedodromous secondary vein were present in four species of *Cleome* viz., *Cleome brachycarpa, C. droserifolia, C. chrysantha* and *C. hanburyana',* semicraspidodromous was restricted only to *Cleome amblyocarpa.* Most taxa had irregular vein spacing and rarely regular, e.g. *Cleome paradoxa,* or increasing towards base as in *Cleome droserifolia.* The second vein angle varied greatly between the studied taxa, so it has no taxonomic value to differentiate between rare taxa. The number of veins on either side of midrib had a significant taxonomic value among different taxa; where *Capparis decidua* was characterized by 7-8 veins on either

Table 2. Characters and character states used in morphometric analysis of Capparaceae.

Character	Character state	Code
1. Leaf or leaflet form	Obovate	1
	Narrow oblong	2
	Orbicular	3
	Ovate	4
	Oblanceolate	5
	Elliptic	6
	Linear	7
2. Leaf apex	Mucronate spiny	1
	Mucronulate	2
	Obtuse	3
	Acute	4
	Retuse	5
	Accuminate	6
3. Leaf margin	Entire	1
	Denticulate	2
4. Leafcomposition	Simple	1
	Trifoliate	2
	Petafoliate	3
5. Leaf surface	Glabrous or pubescent	1
	Glandular hairy	2
	Farinose	3
	Scabrous	4
	Pilose	5
	Viscid	6
6. Leaf base	Cuneate	1
	Acute	2
	Obtuse	3
	Subcordate	4
	Decurrent	5
7. Leaf persistence	Deciduous	1
	Persistent	2
8. Venation pattern	Pinnate	1
	Actinododromous	2
9. Secondary vein category	Brochidodromous	1
	Cladododromous	2
	Craspedodromous	3
	Semicraspedodromous	4
10. Secondary vein spacing	Irregular	1
	Regular	2
	Increasing toward base	3
11. Secondary vein angle	Uniform	1
	Increasing toward base	2
	Decreasing toward base	3
	One pair acute secondaries	4
	Two pair acute secondaries	5
12. Inter-secondary veins	Present	1
	Absent	2
13. Number of veins on either side of midrib	1	1
	4-6	2
	7-8	3
14. Third vein category	Random reticulate	1
	Dichotomous	2
15. Third vein angle to the primary	Acute	1
	Perpendicular	2
16. Fourth vein category	Random reticulate	1
	Dichotomously branched	2
17. Fifth vein category	Absent	1
	Random reticulate	2
	Dichotomously branched	3

Table 2. Continued.

Character	Character state	Code
18. Areolation	5-ormoresided	1
	Poorly or moderately developed	2
19. Free Ending UltimateVeinsofthe leaf (F.E.V.S)	Three branched with swollen dots	1
	One branched tapering	2
	Three branched diffuse	3
	Three branched tapering	4
	Absent	5
20. Attached vein angle	Prismatic	1
	Truncatetriangle	2
21. Marginal ultimate venation	Incomplete loops	1
	Fimbrial arcuate	2
	Fimbrial simple	3
	Excurrentveinbranched	4

side of midrib, *Cleome arabica* and *C. droserifolia* were characterized by one vein on either side of midrib. The third vein category was reticulate and mostly meets the primary veins at acute angles. Quaternary venation was mostly dichotomized branching, while fifth vein category is mostly absent, and, if present, it may be random or dichotomizing branched.

Areolation were usually well developed. In addition, the free ending ultimate veins of the leaf (F.E.V.S.) could be distinguished by having one, two or three branched tapering or swollen ends. Marginal ultimate venation had incomplete loops in genera of *Capparis* (except *Capparis decidua)* and *Maerua;* while fimbrial arcuate was characterized to *Cadaba* and *Boscia.* Branched excurrent vein was characteristic to genus *Cleome,* except *Cleome chrysantha,* which had fimbrial arcuate marginal vein.

Analysis of venation in the Capparaceae indicated that the

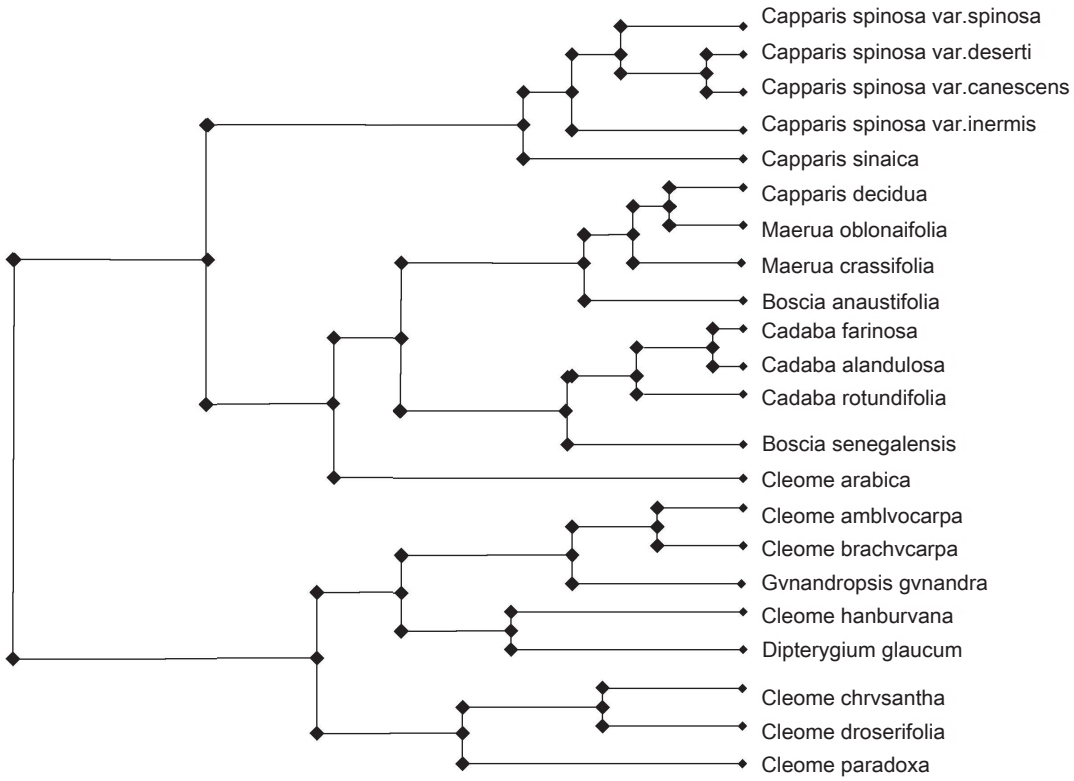

Figure 2. Complete linkage

following characteristics were helpful in the identihcation of the studied taxa: leaf composition, leaf surface, major venation pattern, secondary vein category, number of veins on either side of midrib, areolation, free ending ultimate veins and marginal ultimate venation. Therefore, based on the aforementioned results, the following key can be presented for the identihcation of the Capparaceae:

1. Venation pattern actinododromous
2. Marginal ultimate venation is incomplete loop
Cleome arabica
2. Marginal ultimate venation is branched excurrent vein.....*Cleome droserifolia*
1. Venation pattern pinnate
3. Number of veins on either side of midrib 7-8 *Capparis decidua*
3. Number of veins on either side of midrib otherwise 4
4. Marginal ultimate venation with excurrent vein branched.....5
5. Secondary vein category brochidodromous.....
.....*Cleomeparadoxa*
5. Secondary vein category semi-craspedododromous ……
..... *Cleome amblyocarpa*
5. Secondary vein category cladododromous 6
6. Secondary vein angle decreasing toward base and areolation poorly developed..... *Dipterygium glaucum*
6. Secondary vein angle uniform, areolation well developed 5 or more sided..... *Gynandropsis gynandra*
5. Secondary vein category craspedodoromous 7
7. Marginal ultimate venation hmbrial arcuate and secondary vein spacing increasing toward base.....
..... *Cleome chrysantha*
7. Marginal ultimate venation with excurrent vein branched and irregular secondary vein spacing 8

8. Secondary vein angle with one pair acute secondaries *Cleome hanburyana*
8. Secondary vein angle decreasing towards base .....*Cleome brachycarpa*
4. Marginal ultimate venation with hmrial arcuate 9
9. Free ending ultimate veins (F.E.V.S.) absent 10
10. Secondary vein spacing increasing toward base and secondary vein angle with one pair acute secondaries *Boscia angustifolia*
10. Secondary vein angle with two pair acute secondaries and irregular vein spacing.....
.....*Boscia senegalensis*
9. Free ending ultimate veins with three branched and tapering 11
11. Secondary vein angle decreasing towards base *Cadabafarinosa*
11. Secondary vein angle with one pair acute secondaries *Cadaba glandulosa*
11. Secondary vein angle with two pair acute secondaries *Cadaba rotundifolia*
4. Marginal ultimate venation with incomplete loops 12
12. Free ending ultimate veins with three branched diffuse 13
13. Leaf oblanceolate.....*Maerua crassifolia*
13. Leaf narrow oblong.....*Maerua oblongifolia*
12. Free ending ultimate veins with three branched and swollen dots..... 14
14. Inter-secondary veins absent 15
15. Secondary vein angle uniform.....
.....*Capparis spinosa* var. *deserti*
15. Secondary vein angle increase towards base ....
.....*Capparis spinosa* var. *inermis*
14. Inter-secondary vein present 16
16. Third vein angle to the primary is perpendicular *Capparis spinosa* var. *spinosa*
16. Third vein angle to the primary is acute..... 17
17. Secondary vein angle increasing towards base …….
..... *Capparis spinosa* var. *canescens*
17. Secondary vein angle with two pair acute secondaries *Capparis sinaica*

On the basis of leaf architecture, cluster analysis was used to solve some of the problems met within this family such as: (a) the segregation of *Cleome* species from the *Capparis* group, *i.e.,* into two distinct families or not, (b) whether *Dipterygium* is better placed in Capparaceae than Brassicaceae (Hedge et al. 1980), and (c) the treatment of *Gynandropsis* as separate genus or its restoration as *Cleome gynandra.*

The dendrograms resulted from the cluster analysis are shown in Figures (2-4). Differences between methods arose

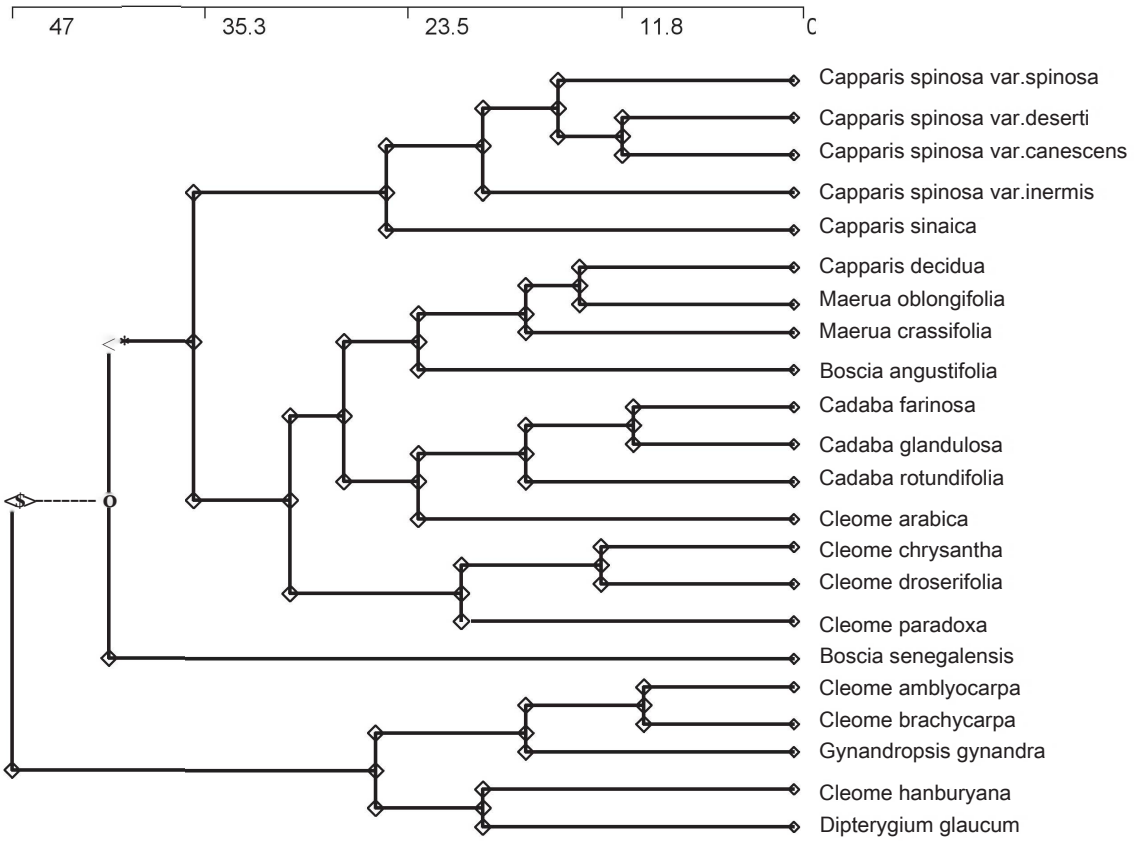

Figure 3. Average linkage

because of the differences in the ways of defining distance (or similarity) between individuals, and a group containing several individuals. All the dendrograms showed that three main clusters can be distinguished: (1) a cluster comprised most of the *Capparis* species; (2) a cluster divided into three subgroups: the first comprises all *Maerua* species, *Capparis decidua* and *Boscia angustifolia,* the second comprises *Cadaba* species and *Boscia senegalensis,* and the third with *Cleome arabica;* and (3) a cluster comprises most of *Cleome* species, *Gynandropsis gynandra* and *Dipterygium glaucum.*

Principal Components Analysis (PCA) reflected which characters were important on the axes; and indicated the significant characters based on the highest factor loading (Table 3). Therefore, it becomes clear which characters caused the separation between groups and can be useful to distinguish taxa. Generally, the results showed congruence between classification and ordination analyses in suggesting the following groups:

1- *Capparis* group (Tribe Capparideae): On the basis of leaf and venation characters, results of PCA confirmed that the studied taxa of *Capparis* formed a well-distinguished group characterized by: (a) simple ovate leaf, (b) irregular secondary vein spacing, (c) random reticulate third vein category and (d) branched free ending ultimate veins with swollen dots.

2- *Boscia* group (Tribe Capparideae): This group characterized by the absence of free ending ultimate veins. Phylogenetically and based on morphological and molecular data, Hall et al. (2002) revealed that there was less supported resolution within the terminal clades of Capparoideae, and still unresolved but comprise five well supported clades.

3- *Cadaba* group (Tribe Capparideae): This group is characterized by: (a) three branched with tapering end of free ending ultimate ends and (b) absence of intersecondary veins. Hall et al. (2002) indicated that genus *Cadaba* is well supported as a natural genus based upon the presence of large adaxial glands in flowers, and thus it can be supported as monophyletic group.

4- *Maerua* group (Tribe Maerueae): This group included the taxa of genus *Maerua.* It is differentiated on the basis of: (a) simple leaf, (b) pinnate venation pattern and (c) three branched diffuse of free ending ultimate veins of the leaf.

5- The mixed group: which included *Cleome* species, *Dipterygium glaucum* and *Gynandropsis gynandra* that characterized by their leaf margin and leaf persistence.

The present results showed some degree of similarity

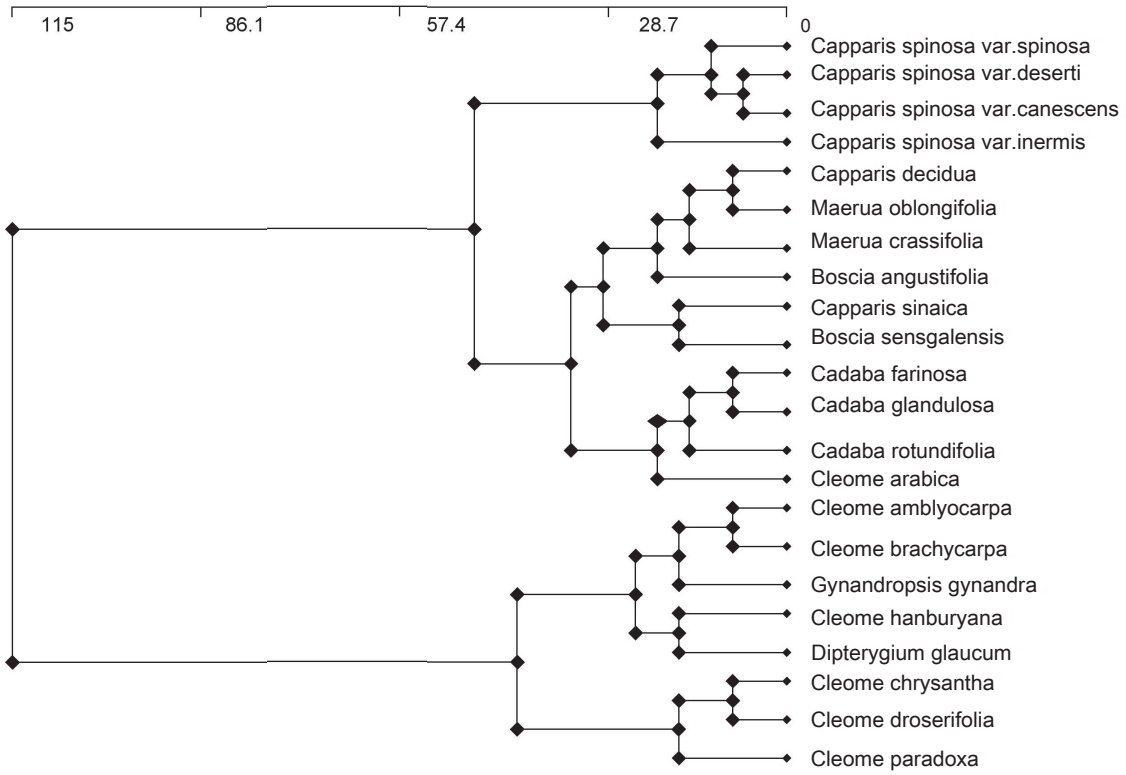

Figure 4. Minimum variance

among the taxa of Dipterygioideae and Cleomoideae based on: (a) the number of veins on either side of midrib and (b) secondary vein category (either cladodromous or craspidodromous).

Table 3 and Figure 5 showed that the main characters explaining this separation were leaf or leaflet form (1), leaf surface (5), leaf base (6), leaf persistence (7), main venation pattern (8), secondary vein angle (11), inter-secondary veins (12), number of veins on either side of midrib (13), free ending ultimate veins (19) and marginal ultimate venation (21).

Discussion

Leaf venation in angiosperm varies both in pattern (Hickey 1973) and regularity (Hickey and Doyle 1972). According to Pray (1954), the veins of first, second and third order form major venation pattern and those of subsequent orders constitute minor venation patterns. Hickey and Wolf (1975) based most of their conclusions on a survey of dicotyledonous leaf architecture made in the course of over ten years' study. They established the first framework for a systematic summary of dicotyledonous leaf architectural features. Because most taxa of dicots possess consistent patterns of leaf architecture, this rigorous method of describing the features of leaves is of immediate usefulness in both modern and fossil taxonomic studies. In addition as a result of this method, it is anticipated that leaves will play an increasingly important part in phylogenetic and ecological studies.

Based on the present study the Capparaceae *sensu lato* manifest two principal types of venation pattern: pinnate and actinododromous. According to Hickey and Wolf (1975), leaves (or leaflets) of the studied taxa of Capparaceae were basically simple, margin entire, venation pinnate, secondary veins were strongly brochidodromous. The free ending ultimate veins of the leaf (F.E.V.S) are a diagnostic character in the Capparaceae. The present observations were in accordance with those of Hickey and Wolf (1975) except the formation of a three-branched free ending ultimate vein with swollen dots (in *Capparis* species, except *C. decidua),* which was not recorded earlier. Whereas it was absent in the studied species of *Boscia,* three branched tapering or diffuse ending was recorded in genera of *Maerua,* and *Cleome arabica.* The remaining *Cleome* species were characterized by one-branched tapering endings.

Cleomoideae and Capparoideae were previously included in Capparaceae (Cronquist 1981, 1988; Thorne 1976, 1983; Dahlgren 1975; Takhtajan 1980, 1976). However, the two subfamilies of Capparaceae (Cleomoideae and Capparoideae) have already been elevated to familial status by some taxonomists (Airy Shaw 1965; Hutchinson 1967). Morphological and molecular studies (Radman 1991a, 1991b; Radman

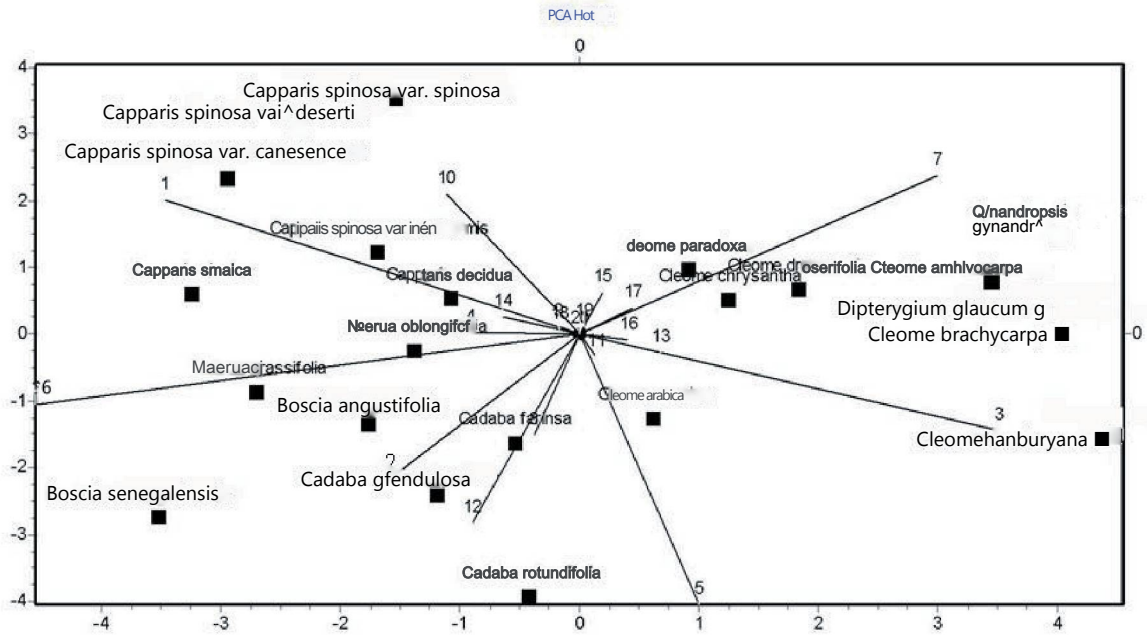

Figure 5. Principal Components Analysis (PCA) biplot showing characters (arrows) and species (dark squares). For character numbers, see Table 2.

et al. 1993; Judd et al. 1994) suggested that Capparoideae form a paraphyletic grade sister to a monophyletic Cleomoideae plus Brassicaceae. Based on these analyses, the two families have been merged into one family: the Brassicaceae *sensu lato* (APG 1998). However; based on molecular data; Cleomoideae, Capparoideae and Brassicaceae all form three well-supported monophyletic clades (Hall and Sytsma 2000; Hall et al. 2002) and could be recognized as three separate families, the Capparaceae, Cleomaceae, and Brassicaceae, a course of action recommended by some recent authors (Hall et al. 2002). On the basis of leaf architecture study, we advocate the recognition of two separate families Capparaceae and Cleomaceae. In this investigation most vein orders of leaf architecture were shown to be of great importance in the taxonomy of Capparaceae and Cleomaceae. They allowed in many instances clear separation between various taxa even at the very lower levels (species level) of the taxonomic hierarchy. Thus, their use to complement macro characters for taxonomic purposes is highly advisable. So for, there is no study devoted to the minor venation pattern, in the Capparaceae and Cleomaceae, the major and minor venation patterns are useful for the identihcation of the species. Our result revealed that leaf architecture has a signihcant value in differentiation between species; however, there is no differentiation at the variety level. We will investigate these tribes and genera with both morphological and molecular data in a separate account.

The present numerical analysis generally in agreement with earlier classification though it has suggested some amendments on generic level. litis (I960), De Wolf (1962), Ernst (1963) and Al-Gohary (1997) adopted the treatment of *Gynandropsis* as separate genus, but this had been contradicted with Tackholm (1974) and Boulos (1999). The cladistic

Table 3. Morphological characters showing highest factor loadings on the first three axes of PCA. For character numbers, see Table 2.

Character Number	Axis 1	Axis 2	Axis 3
1	-1.44	6.25	-1.60
2	-4.36	-2.48	0.058
3	3.28	-0.20	0.61
4	2.12	-1.37	-0.05
5	-0.46	-4.89	-3.07
6	-2.88	-0.61	-0.04
7	-0.45	1.13	-1.31
8	2.88	-0.27	0.95
9	0.56	-2.96	-0.79
10	-0.11	0.42	2.72
11	-5.87	2.21	0.34
12	0.09	0.09	0.24
13	-0.56	1.58	-0.94
14	3.37	0.05	0.83
15	3.02	0.48	0.98
16	-0.21	-0.92	1.04
17	2.55	3.79	-3.68
18	3.00	0.02	0.97
19	-4.52	1.14	248
20	1.94	0.39	1.56
21	-1.92	-3.90	-1.31

analysis of leaf architecture presented in this study supported the concept adopted by some authors as El Hadidi and Fayed (1994/95) and Boulos (1995) who retained *Gynandropsis gynandra* as *Cleome gynandra* of the family Capparaceae, as it clearly nested within *Cleome* (Figures 2-4). Comer (1976) also stated that the seeds of some species of *Cleome* seem to be resemble those of *Gynandropsis* e.g. *Cleome chelidonii.* Our results also added further evidence for the suggestion of Pax and Hoffman (1936), Ernst (1963) and Khafagi and Al-Gohary (1998) that *Gynandropsis gynandra* is closely related to *Cleome hanburyana.*

Although, Täckholm (1974) maintained *Dipterygium* in Cruciferae (Brassicaceae), yet Hedge et al. (1980) and Boulos (1999) favoured better placement in Capparaceae *sensu lato* than in the Cruciferae. This investigation had reinforced evidence for the suggestion of Hedge et al. (1980) for maintaining *Dipterygium* in Cleomaceae. Based on molecular and morphological data, Hall et al. (2002) demonstrated also a strong relationship within the clade including *Cleome* spp., *Dipterygium glaucum* and *Gynandropsis gynandra.*

References

Abd El-Ghani MM, Marei AH (2006) Vegetation associates of the endangered *Randonia africana* Coss. and its soil characteristics in an arid desert ecosystems of western Egypt. Acta Bot Croat 65(l):83-99.

Airy Shaw HK (1965) Diagnoses of new families, new names, etc. for the seventh edition of Willis (Dictionary). Kew Bulletin 18:249-273.

Al Gohary IH (1982) Morphological studies on the Capparidaceae in Egypt. M.Sc. Thesis Botany Department, Faculty of Science, Ain Shams University.

Al Gohary IH (1997) Biosystematic studied of Cleomaceae in Egypt. 1. The seed morphology and its taxonomic significance. Desert Institute Bulletin Egypt 47 (2):423-440.

APG (Angiosperm Phylogeny Group) (1998) An ordinal classification for the families of flowering plants. Annals of the Missouri Botanical Garden 85: 531-553.

APG (2003) An update of the angiosperm phylogeny group classification for the orders and families of the flowering plants. APG II. Botanical Journal of the Linnaean Society 141:399-439.

Banerjee G, Deshpande BD (1973) Foliar venetion and leaf histology of certain members of Compositae. Flora 162:529-532.

Boulos L (1999) Flora of Egypt. Vol. 1 (Azollaceae-Oxalidaceae). Al Hadra Publishing, Cairo.

Boulos L, El-Hadidi MM (1984) The Weed Flora of Egypt. The American university in Cairo Press, Cairo.

Corner EJH (1976) The Seeds of Dicotyledons. Vol.l. Cambridge Univ. Press. Cambridge.

Cronquist A (1981) An integrated system of classification of flowering plants. Columbia University Press, New York, USA.

Cronquist A (1988) The evolution and classification of flowering plants. 2nd Ed. The New York Botanical Garden. New York.

Dalhgren R(1975) A system of classifiaction of the Angiosperms to be used to demonstrate the distribution of characters. Botaniska Notiser 128:119-147.

De Wolf GP (1962) Notes on African Capparidaceae III. Kew Bulletin 16 (l):75-83.

Dilchler DL (1974) Approach to the identification of Angiosperm leaf remains. Bot Rev 40:1-157.

El Hadidi MN, Fayed A (1994/1995) Materials for Excursion Flora of Egypt. Taeckholmia 15:1-233.

El-Karemy ZAR (2001) Capparaceae in the flora of Egypt. Taeckholmia 21(2):257-267.

Endress PK (1992) Evolution and floral diversity: the phylogenetic surroundings *of Arabidopsis wad Antirrhinum.* Int J Plant Sei 153:106-122.

Ernst WR (1963) The genera of Capparacea and Moringaceae in the southeastern United States. Journal of the Arnold Arboretum 44:1-81.

Ettingshausen C von (1861) Die Blattsklete des Dicotyledonen. K.K. Hof. Staatsrnckeri, Wien.

Hall JC, Sytsma KJ (2000) Solving the riddle of California cuisine: phylogenetic relationships of Capers and Mustards. Am J Bot 87(6, Suppl.):132.

Hall JC, Kenneth J, Iltis HH (2002) Phylogeny of Capparaceae and Brassicaceae based on chloroplast sequence data. Am J Bot 89(11): 1826-1842.

Hedge IC, Lamond J (1970) Capparidaceae. In Rechinger K.H. ed., Flora Iranica 68:1-9, Graz.

Hedge IC, Kjaeer A, Malver O (1980) *Dipterygium:* Cruciferae or Capparaceae. Notes from Royal Botanic Gardens Edinburgh 38(2):247-250.

Hickey L (1973) Classification of the architecture of dicotyledonous leaves. Am J Bot 60:17-35.

Hickey L (1977) Stratigraphy and paleobotany of the Golden Valley Formation (Early Tertiary) of western North Dakota. Memoirs of the Geological Society of America 150: 1-183.

Hickey LJ, Doyle JA (1972) Fossile evidence in the evolution of Angiosperm leaf venation. Am J Bot 59:661 (Abstract).

Hickey LJ, Taylor DW (1991) The leaf architecture of *Ticodendron* and the application of foliar character in discerning its relationships. Ann Mo Bot Gard 78:105-130.

Hickey LJ, Wolf JA (1975) The bases of Angiosperm phylogeny: vegetative morphology. Ann Mo Bot Gard 62:538-589.

Hutchinson J (1967) The Genera of Flowering Plants. Univ. Press, Oxford, UK.

Iltis HH (I960) Studies in Capparidaceae VII. Old world Cleomes adventive in the New World. Brittonia 12:279-294.

Inamdar JA, Murthy GSR (1978) Leaf architecture in some Solanaceae. Flora 176:269-272.

Inamdar JA, Shenoy KN, Rao NV(1983) Leaf architecture of some monocotyledons with reticulate venation. Ann Bot52:725-735.

Jacobs M (1965) The genus *Capparis* (Capparaceae) from the Indus to the Pacific. Blumea 12:385-541.

Jain DK (1978) Studies in Bignoniaceae. III. Leaf architecture. J Indian Bot Soc 57:369-386.

Judd WS, Sanders RW, Donghue MJ (1994) Angiosperm pairs: preliminary phylogenetic analyses. Harv Pap Bot5:l-51.

Khafagi A, Al-Gohary IH (1998) Biosystematic studies of Cleomaceae in Egypt. II. Taxonomic significance of some micromorphological characters of the leaf and pollen grains. Al-Azhar Bulletin of Science 19(2):1027-1036.

LAWG (1999) Manual of Leaf Architecture- morphological description and categorization of dicotylenous and net-veined monocotylenomous angiosperms. Leaf Architecture Working Group. Washington.

Levin GA (1986) Systematic foliar morphology of Phyllanthoideae (Euphorbiaceae). 1. Conspectus. Ann Mo Bot Gard 73:29-85.

Li HM, Hickey LJ (1988) Leaf architecture and systematics of the Hamamelidaceae *sensu lato.* Acta Phytotaxonomica Sinica 26:96-110.

Luo Y, Zhou Z-K (2002) Leaf architecture in *Quercus* subgenus *Cyclobalanopsis* (Fagaceae) from China. BotJ Linn Soc 140:283-295.

Mabberley DJ (1987) The plant book. Cambridge University Press, Cambridge, New York.

Mabberley DJ (1997) The plant book: a portable dictionary of the higher plants. Univ. Press, Cambridge, UK.

Pax F, Hoffmann K (1936) Capparidaceae. In Engler and Prantl, eds., Natürlichen Pflanzenfamilien 17(b):146-233.

Pray TR (1954) Foliar venation of Angiosperms. I. Mature venation of *Liriodendron.* Am J Bot 41:663-670.

Rodman JE (1991a) A taxonomic analysis of glucosinolate-producing plants. 1: Phenetics. Syst Bot 16:598-618.

Rodman JE (1991b) A taxonomic analysis of glucosinolate-producing plants. 2: Cladistics. Syst Bot 16:619-629.

Rodman JE, Price RA, Karole K, Conti E, Sytsma KJ, Palmer JD (1993) Nucleotide sequences of the *rbch* gene indicate monophyly of mustard oil plants. Ann Mo Bot Gard 80:686-699.

Shammuka R, Narmada K (1994) Leaf Arcitecture in some Amaranthaceae. Feddes Repertorium 105(l-2):37-44.

Subrahmanyam NS (1999) Laboratory of Plant Taxonomy. Delhi.

Sun H, Chen J, Zhou ZK, Fei Y (1991) The leaf architecture and its taxonomic significance in the genera *Albizia* and *Cylindrokelupha* from China. Acta Botanica Yunnanica 13:241-253.

Täckholm V (1974) Students' Flora of Egypt. Cairo University, Cairo.

Tackhtajan AL (1980) Outline of classification of flowering plants. Bot Rev 46:225-359.

Thakur C (1988) Leaf architecture in Cassia. Acta Botanica Indica 16: 63-72.

Thorn RE (1976) A phylogenetic classification of the Angiospermae. In Hecht MK, Steere WC and Wallace B, eds., Evolutionary Biology 9: 35-106, New York.

Thorn RF (1983) Proposal new realignment in the Angiospermae. Nord J Bot 3: 85-117.

Thulin M (1993) Flora of Somalia. Vol. 1. Royal Botanic Gardens, Kew.

Wang YF, Ferguson DK, Zetter R, Denk T, Garfi G (2001) Leaf architecture and epidermal characters in *Zelkova* (Ulmaceae). Bot J Linn Soc 136: 255-265.

Wieringa JJ (1999) Monopetalanthus exit. A systematic study of *Aphanocalyx, Bikinia, Icuria, Microberlinia* and *Tetraberlinia* (Leguminosae, Caesalpinioidae). Wageningen Agriculture University Papers 99(4):l-320.

Zahran MA, Willis AJ (1992) The Vegetation of Egypt. Chapman and Hall, London, UK.

Zohary M (1966) Flora Palestina.Vol.l, Israel Academy of Sciences and Humanities, Jerusalem.

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DISSERTATION SUMMARIES

Photoprotection difficulty in D1 protein mutant of *Solanum nigrum*

Szilvia Bajkań

Department of Botany, University of Szeged, Szeged, Hungary

Plants are usually exposed to wide ranges of variable and fluctuating light intensities. The excess light energy may damage the photosynthetic apparatus. One of the most important and rapid photoprotective mechanisms is the thermal dissipation of light energy, referred to in the literature as non-photochemical quenching (NPQ) of chlorophyll fluorescence. The major component of NPQ is ApH-dependent, rapidly reversible, and called qE quenching. Its formation requires low thylakoid lumen pH, de-epoxidized xanthophylls (Demmig-Adams et al. 1996; Horton 1996; Eskliing et al. 1997), and PsbS protein (Li et al. 2000).

Naturally selected atrazine-resistant (AR) weeds in crop cultivation have a modified D1 protein structure, with a Ser_{264}^ Gly (S264G) mutation near the Q_B niche (Hirsberg and McIntosh 1983). This mutation greatly reduces the affinity of Q_B and atrazine for Q_B-binding pocket, and causes higher susceptibility to photoinhibition in several weed species (Hart and Stemler, 1990; Varadi et al. 2003).

This summary addresses the question of whether the conserved chloroplastic DNA encoded D1 protein is required for normal photoprotection of plants or not. In order to ascertain how the photoprotective thermal dissipation functions are influenced by the S264G D1 protein mutation (which is related to atrazine resistance), experiments were performed on whether qE is controlled only by nuclear, or by both nuclear and cytoplasmic factors. Photosynthetic properties, chlorophyll fluorescence quenching-related parameters and xantophyll cycle activity were compared in different S. *nigrum* lines.

We used two inbred lines of *Solanum nigrum:* Wild type, atrazine sensitive (AS) and atrazine resistant (AR); *i.e.* carrying the S264G D1 mutation in its chloroplastic DNA. To generate FI hybrids between the two parental lines, we set up reciprocal crosses between AS and AR parents. The heterozygous FI plants were allowed to self-pollinate to generate F2 seeds, and designated then as ARF2 and ASF2. The presence of S264G mutation of different *S. nigrum* lines was monitored by chlorophyll fluorescence induction of intact leaves by using the characteristic F_i/F_m fluorescence parameter. The modulated chlorophyll *a* fluorescence was measured on 30-min dark-adapted attached leaves, using a Dual Channel Modulated Fluorimeter (Hansatech, England). Xanthophyll cycle components were determined by means of HPLC according to Vaŕadi et al. (1992).

The fast chlorophyll fluorescence induction kinetics of parents and hybrid lines of *S. nigrum* in principle showed similar induction transients. As expected however, the leaves containing AR chloroplastic genome (AR, ARF1 and ARF2 plants) were always characterized by significantly higher intermediate F_i chlorophyll fluorescence level as compared to the AS, ASF1 and ASF2 wild types. Light-induced gross NPQ for the wild biotypes normally reaches a value of 2.5 to 3, but it remained between 1 and 1.5 (mainly around 1) in the AR biotypes, *i.e.* decreased by about 50%. The capacity of the xanthophyll cycle is lower by 20% in the mutant biotypes. The comparison of Zea and pH dependent qE non-photochemical quenching, determined as the rapidly relaxing component of NPQ showed more definite differences between the wild and D1 protein mutant weeds. The investigated S264G D1 protein mutant lines exhibited 60-70% reduced values of qE. The results of this research clearly show that the PsbA gene-encoded highly conserved D1 protein structure, found in the wild type plants, is also essential for qE photoprotective chlorophyll fluorescence quenching, as revealed by maternal inheritance of D1 protein mutation and reduced qE level.

Demming-Adams B, Adams WW, Barker DH, Logan BA, Bowling DR, Verhoeven AS (1996) Using chlorophyll fluorescence to assess the fraction of absorbed light allocated to thermal dissipation of excess excitation. Physiol Plant 98:253-264.

Eskling M, Arvidsson P-O, Akerlund H-E (1997) The xantophyll cycle, its regulation and components. Physiol Plant 100:806-816.

Hart JJ, Stemler A (1990) High light-induced reduction and low light-enhanced recovery of photon yield in triazine-resistant Brassica napus L. Plant Physiology 94: 1301-1307.

Hirsberg J and McIntosh L (1983) Molecular basis ofherbicide resistance in Amaranthus hybridus. Science 224: 1346-1348.

Horton P, Ruban AV, Walters RG (1996) Regulation of light harvesting in green plants. Ann Rev Plant Physiol Plant Mol Biol 47:655-687.

Li X-P, Bjoŕkman O, Shis C, Grossman AG, Rosenquist Jansson S, Niyogi KK (2000) A pigment-binding protein essential for regulation of photosynthetic light harvesting. Nature 403:391-395.

Váradi Gy, Botos-Baló B, Polös E (1992) Xanthophyll cycle in grapevine leaves - diurnal and seasonal patterns. Proceedings of the IVth International Symp on Grapevine Physiology, 11-15 May. San Michele All'adige, Torino, pp. 521-526.

Váradi Gy, Polyańka H, Darko É, Ĺehoczki E (2003) Atrazine resistance entails a limited xanthophyll cycle activity, a lower PSII efficiency and an altered pattern of excess excitation dissipation. Physiol Plant 118:47-56.

Supervisor: Endre Lehoczki
E-mail: szilviabajkan@yahoo.com

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Identification of Hox2, the second NAD$^+$-reducing NiFe hydrogenase in the purple sulfur photosynthetic bacterium *Thiocapsa roseopersicina* BBS

Judit Balogh

Institute of Biophysics, Biological Research Center, Hungarian Academy of Sciences, Szeged, Hungary, Department of Biotechnology, University of Szeged, Szeged, Hungary

Hydrogenases are metalloenzymes catalyzing the oxidation of molecular hydrogen and also the reverse reaction, the evolution of hydrogen. Since, hydrogen has been reported as a good energy carrier and Sun as an environmentally friendly energy source, the study of these enzymes in photosynthetic microorganisms is a hot subject nowadays.

Thiocapsa roseopersicina BBS is a purple sulfur photosynthetic proteobacterium belonging to the Chromatiaceae family. This preferentially anaerobic bacterium, capable to grow under several different conditions (anaerobic, micoraaerobic conditions, ligth, dark, nitrogen fixing), is able to use different electron donors as reduced sulfur compounds and simple organic substrates.

The presence of at least three functional hydrogenases (HupSL, HynSL and HoxEFUYH) (1,2,3) have been reported in this bacterium. Deletion of all the mentioned H_2ases led to a strain still having hydrogenase activity. *T. roseopersicina* genome project is going on. Part of a contig of the sequenced gDNA showed homology to *hoxH* genes encoding for the large hydrogenase subunit of the bidirectional heteromultimeric cytoplasmic [NiFe] hydrogenases. Since a Hox type hydrogenase has already been identified in *T. roseopersicina* the new gene was named *hoxH2*.

A 6 kb DNA fragment, containing the *hoxH2* gene, was cloned and further *hox2* genes were identified. The *hoxY2* encodes for the hydrogenase small subunit, *hoxF2* and *hoxU2* code for diaphorase subunits. In the genome of *Thiocapsa roseopersicina* BBS a region showing homology to *hoxW* genes was also identified, which likely encode for endoproteases responsible for the cleavage of the C-terminal extension of the large subunit as the final step of the processing.

Hydrogenase activity measurements were done using the GB112131 *(hynSL, hupSL* and *hoxH* mutant) strain grown on various media and *in vivo* uptake and evolution activity were detected under certain conditions. These results suggest the presence of a heterotetrameric NAD$^+$-reducing bidirectional soluble NiFe hydrogenase, Hox2 in *T. roseopersicina.* The existence of two Hox enzymes in a single cell is unique for this bacterium and may disclose further diversification of the Hox enzymes.

Rakhely G, Colbeau A, Garin J, Vignais PM, Kovacs KL (1998) Unusual organization of the genes coding for HydSL, the stable [NiFe]hydrogenase in the photosynthetic bacterium Thiocapsa roseopersicina BBS. J Bacteriol 180:1460-1465.

Kovacs KL, Kovacs AT, Maroti G, Meszaros LS, Balogh J, Latinovics D, Fulop A, David R, Doroghazi E, Rakhely G (2005) The hydrogenases of Thiocapsa roseopersicina. Biochem Soc Trans 33:61-63.

Supervisors: Kornél L. Kovács, Gábor Rákhely
E-mail: baloghj@brc.hu

Effect of heavy metal stress at molecular, cellular and whole plant level and the stress responses in plants

Bernadett Bartha

Department of Plant Biology, University of Szeged, Szeged, Hungary

Heavy metals are important environmental pollutants with high toxicity to animals and plants. Cadmium, copper and zinc in high concentrations damage plants by causing oxidative stress, modulating the uptake and distribution of other essential macro elements, inactivate or denature proteins by binding to sulfhydryl groups and degrading the photosynthetic apparatus (Schützendübel and Polle 2002). Higher plants exhibit considerable variation in their ability to tolerate bivalent cation contamination in their environment. Several tolerant species can accumulate significant levels of these metal ions, while another sensitive species can be impaired by lower metal concentrations. Heavy metals can be detoxified by different mechanisms: conjugation with phytochelatins, by scavengers or using the antioxidant enzyme capacity of plants. In this enzymatic way the catalase (CAT), guaiacol peroxidase (GPX), superoxid dismutase (SOD), and glutation reductase (GR) are involved (Zenk 1996).

In our experiments two plant species were investigated: the heavy metal tolerant- and accumulator Indian mustard *(Brassicajuncea L.* Czem.) and the sensitive agricultural species pea *(Pisum sativum* F.).

We compared the different physiological changes in the two plant species such as heavy metal accumulation, enzymatic responses and growth parameters. Our aim is to reveal the characteristic properties of metal tolerant plants which can be useful in biological soil cleaning technologies such as phytoremediation (Salt et. al. 1995).

Plants were grown in Hoagland nutrient solution under controlled conditions. Cd, Cu or Zn salts were added in different concentrations to the nutrient solution. We measured the capacity of the heavy metal uptake and accumulation, the root-shoot translocation, and the changes of the macro element distribution. All plants accumulated significant levels of the applied metal ions in their roots, however,

Brassica accumulated Zn ions preferentially in the leaf tissue. Copper treatment had a particularly drastic effect on the root system of both species. The effects were plant- and heavy metal specific: in general in Indian mustard the responses for heavy metal stress were the moderate decreases of oxidative enzyme activities while in pea the activities showed slight increases. The most significant changes in enzyme activities occurred in the second and third days after treatment in both species.

Literature data suggest that there is a causal relationship between NO and metal ion metabolism (Neill et. al. 2002). Our aim is to demonstrate the possible role of NO in the plant response to heavy metals. After treatments, the NO appearance was measured in the root tips with fluorescent method, using a very specific dye to nitric oxide, diaminofluorescein-diacetate (DAF-2 DA). We obtained different NO levels with different heavy metal loads: the most effective metals were copper and cadmium, in this case the NO production became double after one week treatment. In case of copper load, we found a fast NO burst in the first six hours (Bartha et. al. 2005). This fast appearance and the good mobility of NO suggest that it can be a signal molecule in plants under heavy metal stress.

Compared the two species we found basic differences in their cadmium detoxification mechanisms and oxidative stress defense responses. These experiments can help us to find the physiological background of the heavy-metal tolerance. On the basis of our results phytoremediation technology will be worked out. Our results are already applied in pilot experiments on polluted environment and our further aim is to work out a complete phytoremediation technology (Vashegyi et. al. 2005).

Bartha B, Kolbert Zs, Erdei L (2005) Nitric oxide production induced by heavy metals in Brassica juncea L. Czem. and Pisum sativum L. Acta Biol Szeged 49:9-12.

Neill SJ, Desikan R, Clarke A, Hurst RD, Hancock JT (2002) Hydrogen peroxide and nitric oxide as signalling molecules in plants. Journal of Experimental Botany 53:1237-1247.

Salt DE, Blaylock M, Kumar NPBA, Dushenkov V, Ensley BD, Chet I, Raskin I (1995) Phytoremediation: a novel strategy for removal of toxic metals from the environment using plants. Biotechnology 13:468-474.

Schützendübel A, Polle A (2002) Plant responses to abiotic stress: heavy metal-induced oxidative stress and protection by mycorrhization J Exp Bot 53(372):1351-1365.

Vashegyi Á, Mezősi G, Barta K, Farsang A, Dormány G, Bartha B, Pataki Sz, Erdei L (2005) Phytoremediation of heavy metal pollution: A case study. Acta Biol Szeged 49:77-79.

Zenk MH (1996) Heavy metal detoxification in higher plants - a review. Gene 179:21-30.

Supervisor: László Erdei
E-mail: barthab@bio.u-szeged.hu

Comparative analysis of 10th -11th century populations in the southern part of the Great Hungarian Plain. A preliminary report

Zsolt Bereczki

Department ofAnthropology, University of Szeged, Hungary

The regional anthropological characteristics of the Carpathian Basin have never been uniformal, so the biological effects of the Hungarian Conquest were versatile, too. However, in some regions of the country late Avar populations show quite a lot of similarities with early Árpádian Age populations in general (Szathmáry 1996). This fact might be explained by the possible survival of autochthonous groups from the 9th century.

Compared to other regions of the country, Late Avar Age populations and ethnic groups of the Age of the Hungarian Conquest in the southern part of the Great Hungarian Plain show less differences (Barabás et al. 1996), although, new settlers are known to have arrived here during the Conquest. For this reason, the area seems to be a best investigation area concerning survival of certain populations.

The aim of our study is to perform a complete comparative analysis of cemeteries from the lC7h and 11th century in the southern part of the area east of river Tisza using both classical methods of historical anthropology and statistical analyses of metrical data. The following series were included in our study: Békés-Povádzug 10th-12th c., Biharkeresztes-Ártánd-Nagyfarkasdomb lC7h-llth c., Eperjes-Ifjú Gárda Tsz. 11th c., Gyula-Fövenyes llth-16th c., Hódmezővásárhely-Nagysziget 10th-llth. c., Kiszombor-B 10th-llth c., Magyarhomorog-Kónyadomb 10th-12th c., Orosháza-Rákóczitelep 10th-12th c., Sarkadkeresztúr-Csapháti-legelő lC7h-llth c., Sárrétudvari-Hízóföld 10th c., Szegvár-Oromdűlő 10th-llth c., Szegvár-Szőlőkalja 10th c. The sample consists of the remains of 2090 individuals (668 male, 620 female, 215 undetermined, 587 subadult). In order to facilitate our biostatistical examinations in the future, we had to choose series with as many cases as possible. Thus, only cemeteries containing at least 50 graves were included in our study. These series usually belonged to the common people of the era and do not represent the whole society. Another concern of sampling was not to leave out any important 10th or 11th century skeletal population. This condition forced us to include series containing lot of burials from the Arpádian Age.

The data of the formerly published series we took over from the references. In case of our own examinations (Eperjes-Ifjú Gárda Tsz., Hódmezővásárhely-Nagysziget (Bereczki et al. 2003a, 2003b), Sarkadkeresztúr-Csapháti-legelő (Bereczki and Marcsik 2005), Szegvár-Szőlőkalja) we used commonly accepted methods of historical anthropology. In some cases X-ray pictures were taken to support pathological diagnoses. All bone material and documentation is housed at the Department of Anthropology, University of Szeged.

Our investigation is long not completed yet, at the current state of the process we can only present our preliminary results. Having successfully finished the examinations we will aim to give answers to questions like e. g. what kind of biological relation exists between these

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populations not far from each other in space and time; in which area was the survival of late Avar populations possible; and what kind of biological impact did the time period before and after the Foundation of State have. Besides, our study provides important new data to the prevalence of certain trephination techniques and metastatic tumors.

Barabás K, GubaZs, Szathmáry L, Loríńczy G (1996) Avar kori népesség-honfoglalás kori népesség. In Pálfi Gy, Farkas L Gy, Molnár Eszerk.: Honfoglaló magyarság-Árpád kori magyarság, Szeged, 1996, pp. 79-86.

Szathmáry L (1996) Honfoglalás kori népességünk struktúrája. In Pálfi Gy, Farkas L Gy, Molnár E szerk.: Honfoglaló magyarság-Arpád kori ḿagyarság, Szeged, 1996, pp. 87-96.

Bereczki Zs, Marcsik A, Paja L (2003a) New cases of trephination from a 10-llth century Hungarian site. Papers on Anthropology 12 (2003), Univ. of Tartu, Estonia, pp. 21-31.

Bereczki Zs, Paja L, Marcsik A, Molnár E (2003b) Rosszindulatú csontdaganatok oszteoarcheológiai szériákban az Alföld területérol-Esetlefírások; A MBT 3. Kárpát-med. Bioi. Szimpóziuma, 2003. okt. 28-30., Konferenciakötet, pp. 317-320.

Bereczki Zs, Marcsik A (2005) Trephined skulls from ancient populations in Hungary. Acta Med Lithuanica 12(l):65-69.

Supervisor: Antónia Marcsik
E-mail: bereczki.zsolt@bio.u-szeged.hu

Cyclin C and the development of mice

Péter Blazsó

Institute of Genetics, Biological Research Center, Hungarian Academy of Sciences, Szeged, Hungary

Cyclins are a group of proteins in eukaryotic cells involved mainly in the regulation of cell cycle. Mammalian and *Drosophila melanogaster* cyclin C genes (Katona and Lahti 2006) were originally identified by rescuing a *Saccharomyces cerevisiae* strain that carried mutation in all known Gl-type cyclins (CLN1, 2 and 3). Later studies did not support the assumption that cyclin C could have been a Gl-type cyclin. In further experiments it was shown that cyclin C was a regulator of mRNA transcription through different mechanisms (Loyer et al. 2005). In higher eukaryotes cyclin C and Cdk8 together are responsible for the phosphorylation of proteins associated directly or indirectly with mRNA synthesis. Cdk8 with cyclin C attached phosphorylates cyclin H inhibiting its transcriptional and cell cycle function through Cdk7 and TFIIH. Moreover, together cyclin C and Cdk8 phosphorylate the carboxy terminal domain (CTD) of the large subunit of RNA polymerase II and they hyperphosphorylate and inactivate Notch negatively regulating its effect on mRNA transcription. Cyclin C and Cdk8 proteins are also part of one of the mammalian Mediator complexes. These complexes were demonstrated to promote transcription *in vitro*. It has also been revealed that cyclin C cooperates with Cdk3. This molecule complex has been thought to play a role in the exit from GO phase by phosphorylating specific residues of Retinoblastoma protein (pRb) (Ren and Rollins 2004). Cyclin C was demonstrated to interact functionally with c-myc, which promotes cell division. Overexpression of cyclin C elevates the level of cdc2 mRNA, a key player in G2/M transition (Liu et al. 1998). Growing number of other evidences suggest that cyclin C acts not only in mRNA transcription but in cell cycle regulation as well in mammalian cells.

Preliminary experiments pointed out that cyclin C may have a vital role in early embryogenesis of mice *(Mus musculus)*. Delation of the transcriptign, translation start point and the first four exons of cyclin C gene results in an embryonic lethal phenotype. Cyclin C mice die before 10 day (E10) of embryonic development (Katona et al. unpublished data). Body sizes are significantly smaller in cyclin C knock-out embryos than in their heterozygous or wild-type littermates. Cyclin C null mutants suffer from serious abnormalities. The cranial end remains split. Heart is large relative to body and unstructured. Forelimbs are usually vestigial and no rear limbs can be found. Generally, the caudal part of the body does not develop at all. Serious retardation can be seen in the differentiation and growth of many other tissues as well. The placental labyrinthine layer, yolk sac and potentially the embryo itself are poorly vascularized.

These findings raise the following questions. What is the tissue localization of cyclin C in early embryonic development? How does the cyclin C expression pattern correlates with the experienced knock-out phenotype?

In order to address these questions we generated transgenic mice carrying a hrGFP reporter gene construction driven by an 3,5 kb upstream region (putative promoter) of the mouse cyclin C gene.

Katona RL, Lahti JM (2006) Cyclin C. AfCS-Nature Molecule Pages. doi:10.1038/mp.a000720.01.

Loyer P, Trembley JH, Katona R, Kidd VJ, Lahti JM (2005) Role of CDK/cyclin complexes in transcription and RNA splicing. Cell Signal 17:1033-1051.

Ren S, Rollins BJ. (2004) Cyclin C/cdk3 promotes Rb-dependent GO exit. Cell 117:239-251.

Liu ZJ, Ueda T, Miyazaki T, Tanaka N, Mine S, Tanaka Y, Taniguchi T, Yamamura H, Minami Y (1998) A critical role for cyclin C in promotion of the hematopoietic cell cycle by cooperation withc-Myc. Mol Cell Biol 18:3445-3454.

Supervisor: Róbert Katona
E-mail: blazso@brc.hu

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Integrative approach of Photosystem II repair in Synechocystis PCC6803 under UV-B damage: proteases, prohibitins and sHSP

Otilia Cheregi

Photobiology and Molecular Stress Group, Institute of Plant Biology, Biological Research Center, Szeged, Hungary

Cyanobacteria, the most widespread and abundant oxygenic photosynthetic prokaryotes, are exposed to various types of environmental stresses. With the recent thinning of the ozon layer, the UV-B component of solar radiation is of particular importance. Its targets inside the PSII are the water oxidizing complex (Vass 1999) and the D1 and D2 proteins (Friso et al. 1994; Friso et al. 1995). In intact photosynthetic organism the structure and function of photodamaged PSII centers can be repaired. The critical step of this repair process is the removal of damaged D1 and D2 proteins, followed by the novo synthesis of the D1 and D2 subunits and reassembly and reactivation of the PSII complex.

We have investigated the role of FtsH and Deg proteases in the degradation of UV-B damaged PSII reaction centers proteins D1 and D2 in Synechocystis 6803. We analyzed also the possible involvement of prohibitins in the repair cycle.

PSII activity in a AFtsH (slr0228) strain, showed increased sensitivity to UV-B radiation and impaired recovery of activity in visible light after UV-B exposure.

In contrast, in ADeg cells, in which all the three deg genes were inactivated, the damage and recovery kinetics were the same as in WT. Immunoblotting showed that the loss of both the D1 and D2 protein was retarded in FtsH (slr0228) during UV-B exposure, and the extent of their restoration during the recovery period was decreased relative to the WT. However, in the Deg cells the damage and recovery kinetics of D1 and D2 were the same as in the WT. These data demonstrate a key role of FtsH (slr0228), but not Deg proteases, for the repair of PSII during and following UV-B radiation at the step of degrading both the UV-B damaged D1 and D2 reaction center subunits.

The genome of Synechocystis contains 5 genes for putative prohibitin proteins. Due to the formation of protein complexes containing FtsH protease and prohibitins in mithocondria of S. cerevisiae and E. coli, a similar interaction was investigated in Synechocystis. Silva and Nixon (unpublished) showed that His-tagged PSII contain both FtsH and prohitin (PhBl).

PSII activity in a triple prohibitin mutant (AT) and a quadruple one (AQ) measured as the rate of oxygen evolution, under UV-B alone or combined illumination: UV-B and VIS, did not show a significant difference as compared with the WT cells.

Synechocystis PCC 6803 has one small heat shock protein Hspl7 that is known to protect thylakoid membrane against heat and photoinhibitory effects of light exposure (Balogi et al. 2005). Two point mutations in Hspl7, L9P and Q16R resulted in dramatic changes in the cellular distribution of Hspl7 in heat/light acclimated cells: an important fraction of L9P was found in the cytoplasm, while Q16R was exclusively associated with the thylakoid membrane (Balogi, unpublished results). Under UV-B stress the heat/light hardened cells behaved differently: WT and L9P cells lost their photosynthetic activity and the Q16R mutant protected almost completely the photosynthetic function (measured as the rate of oxygen evolution).

Balogi Z, Torok Z, Balogh G, Josvay K, Shigapova N, Vierling E, Vigh L, Horvath I (2005) Heat shock lipid in cyanobacteria during heat/light-acclimation. Arch Biochem Biophys 436:346-354.

Friso G, Barbato R, Giacometti GM and Barber J (1994) Degradation of D2 protein due to UV-B irradiation of the reaction centre of photosystem II. FEBS Eett 339: 217-221.

Friso G, Vass I, Spetea C, Barber J and Barbato R (1995) UV-B-induced degradation of the D1 protein in isolated reaction centres of Photosystem II. Biochim Biophys Acta 1231:41-46

Vass I, Kirilovsky D and Etienne A-E (1999) UV-B radiation induced donor and acceptor side modifications of Photosystem II in the cyanobacterium Synechocystis PCC6803. Biochemistry 38:12786-12794.

Supervisor: Imre Vass
E-mail: ocheregi@brc.hu

Regulation of translesion synthesis

Andrea Daraba

Institute of Genetics, Biological Research Center, Hungarian Academy of Sciences, Szeged, Hungary

Cellular DNA is continually damaged by a variety of sources, including UV light from the sun and reactive oxygen species resulting from aerobic respiration. Although cells posses a variety of repair processes to remove DNA lesions, lesions that escape repair can block the replicational machinery. Eukaryotic cells overcome such blocks and promote the continuity of the newly replicated DNA strand through translesion DNA synthesis.

Genetic studies in the yeast Saccharomyces cerevisiae have indicated that Rad6 an ubiquitin-conjugating enzyme together with Radl8 a DNA-binding protein controls the bypass of UV-damaged DNA via at least three separate pathways: an error-free pathway dependent on the RAD5, MMS2 and UBC13 genes, another error free pathway dependent on the RAD30 gene, and a third pathway that is mutagenic and dependent on the REV1, REV3 and REV7 genes.

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Since Rad6-Radl8 complex is indispensable for the function of all three DNA damage pathways the question was what would be the mechanism through which Rad6-Radl8 regulates these pathways.

It has been published that Rad6 together with Radl8 monoubiquitylates PCNA, which serves as a sliding clamp for the replicative polymerases. Two other members of the Rad6 group, Ubcl3 and Mms2, form a heterodimeric ubiquitin-conjugating enzyme, which together with the RING-finger-containing protein Rad5 are responsible for the polyubiquitylation of PCNA.

We wanted to identify other possible substrates Rad6-Radl8 might have. First we checked whether Rad5 could be a substrate of Rad6-Radl8. Using purified proteins we have performed in vitro ubiquitylation of Rad5 using Rad6-Radl8. Our results showed that Rad6-Radl8 efficiently ubiquitylates Rad5 in vitro. To check the in vivo significance of Rad5 ubiquitylation we chromosomally tagged Rad5 with 6His3Ha tag. We treated the cells with the DNA damaging agent MMS and we prepared whole cell extract and performed western blot using a-HA antibody to detect Rad5 and any modified forms of Rad5. Indeed we could detect two higher molecular weight forms of Rad5. According to the molecular weight one could be the ubiquitilated form of Rad5. Experiments using specific anti ubiquitin antibody to verify our result are still in progress.

We also want to determine which Lysine residue is involved in the ubiquitylation of Rad5. We have sent in vitro ubiquitilated Rad5 to mass spectroscopy analysis, but unfortunately we could not get back any positive results. To overcome this problem we have created C terminal deletion mutants of Rad5 to map the region that involves the ubiquitilated lysine residue. We have cloned, overexpressed and purified these mutants and presently we are checking which one of them can be ubiquitylated in vitro. Once the target lysine is found genetic analysis will be performed to see the effect of this mutation on DNA damage bypass.

Supervisor: Ildiko Unk
E-mail: andreea@brc.hu

Study of the mitochondrial DNA polymorphism among Candida species

Zoltán Farkas

Department of Microbiology, University of Szeged, Szeged, Hungary

Recently the number of infections caused by Candida species increased significantly. Localized, invasive or systemic infections (candidiasis) are frequently associated with immune deficiencies, antibiotic treatment, immunosuppressive therapy and various invasive medical procedures (Nősek et. al. 2002, Deák et. al. 2004). The most frequent species isolated from Candida-infected humans is *C. albicans,* however, the occurrence of other species e.g. *C. glabrata, C.parapsilosis, C. krusei, C. tropicalis* increased as well.

The size, organization and gene content of the mitochondrial DNA in yeasts display high degree of variety. Therefore study of the mitochondrial genomes may give deeper insight into the genetic relatedness of the species. However mitochondrial DNA is typically described as a circular molecule, there are examples in the genera Candida, which have linear mtDNA, with telomeres on the ends of the DNA. Moreover the form of mitochondrial genome may differ within the same species, like in *C. metapsilosis.* Some experiments suggest that, under some circumstances, the linearity and/or the presence of telomeres provides a competitive advantage over a circular-mapping mitochondrial genome (Nősek et. al. 2004).

C. albicans has a circular mitochondrial genome with 40420 bp as confirmed by DNA sequencing. In the present study we analysed the mtDNA polymorphism among 44 clinical isolates of *C. albicans.* All the strains with one exception derived from hemo-culture. Four types of mtDNA were detected on the basis of the RFLP pattern of the ffiw/I-digested total DNA. The most frequented type was labelled as number I. The group number IV was represented only by one isolate. The restriction profile of the purified mtDNA confirmed the existence of these types. Interestingly the *Pvull* digestion revealed the same RFLP pattern in each type. Other enzymes (EcoRV, *BglU)* gave distinct result what means that the different mtDNA types derived from point mutations or recombination.

Despite *C. albicans, C. parapsilosis* has linear mitochondrial DNA with tandem repeats at the termini (Nősek et. al. 2004). Earlier study demonstrated the very stable RFLP pattern of the mtDNA within group I isolates of *C. parapsilosis.* Our survey on Hungarian clinical isolates confirmed these results (Pfeiffer et al. 2006).

An international project was established to study the taxonomic relationship and the evolution of the mitochondrial genome in the clade involving *C. parapsilosis* (Diezmann et al. 2004) and some closely related species, such as *C. metapsilosis, C. orthopsilosis* (Kosa et al. 2006), *C. albicans* (Anderson et al. 2001), *C. tropicalis, C. dubliniensis, C. sojae* and *C. maltosa.* In the frame of this work our aim was to get the whole sequence of the mtDNA of *C. maltosa.* Firstly, we have made the restriction map of the mtDNA of *C. maltosa* using the restriction endonucleases *Eco¥N* and *Pstl.* We have made random sequencing after physical breaking (nebulization) of the mtDNA. The sequences were aligned by software generating contigs by overlapping regions of the sequences. We already have the 70 percentage of the mitochondrial genome, involving genes of nadl, nad4, nad5, coxl, cox3a, cox3b, cob, ml, atp6 and some tRNA. The remaining gaps will be filled with primer walking in the near future.

Anderson JB, Wickens, Khan M, Cowen LE, Federspiel N, Jones T, Kohn LM (2001) Infrequent genetic exchange and recombination in the mitochondrial genome of Candida albicans J Bacteriol 183(3):865-872.

Deák R, Bodai L, Aarts HJ, Maráz A (2004) Development of a novel, simple and rapid molecular identification system for clinical Candida species. Medical Mycology 42(4):311-318.

Nősek J, Novotna M, Hlavatovicova Z., Ussery DW, Fajkus J, Tomaska L (2004) Complete DNA sequence of the linear mitochondrial genome of the pathogenic yeast Candida parapsilosis. Mol Gen Genomics 272:173-180.
Nősek J, Tomaska L, Rycovska A, Fukuhara H (2002) Mitochondrial telomeres as molecular markers for identification of the opportunistic yeast pathogen Candida parapsilosis. J. Clin. Microbiol. 40(4):1283-1289.
Pfeiffer I., Litter J., Farkas Z., Kucsera J (2006) Élesztőgombák mitokondriális DNS szerveződésének vizsgálata. A Magyar Tudomány Ünnepe Szegeden. Szeged, Magyarország.

Supervisor: Ilona Pfeiffer
E-mail: cheeroke@freemail.hu

Children growth in the past

Ágnes Fogl

Department ofAnthropology, University of Szeged, Szeged, Hungary

Children growth is a separate area in physiology and anthropology. Growth rate can be examined using auxology studies. The main aim of auxology is to recognize the principles of growth on the base of longitudinal or mixed sampling (Farkas 2000).

Complications can occur during studying archaeological samples, especially when working with children samples. Very often bones are fragile, and there are difficulties with determining age at death and sex.

Chronological age can be estimated by using biological age-markers at their developmental stage.

One of these age-markers is the development of the skeleton that can be estimated by epiphyseal growth or long-bone measures. In the case of children, whose epiphysis has not closed yet, measurements were used as suggested by Fazekas and Kósa (1978). If the epiphysis was closed, measurements were used as suggested by Knussmann and Martin (1988).

Another method for estimating age is detecting teeth-eruption through different developmental stages (Schour and Massler, 1941).

In both cases it should be taken into consideration that environmental stresses (nutrition, diseases, etc.) have certain effect on bone-measurements and teeth-eruption time. Bones are much more affected then teeth.

Visser (Visser 1998) created regression equation for stature estimation on the base of radiographs made by Maresch in 1943. These equations need the measurements of the humerus, femur and tibia.

The main aim of this study was to get information on children growth by the examination of length and diameter of the long bone's diaphysis in the age categories defined by teeth eruption-time. After mathematical-statistical analysis conclusions were to be reached about children growth in the Avarian age. As a last step the Avarian data were compared with contemporary children data.

As material the Bélmegyer-Csömöki domb cemetery from the Avarian age was studied. Archaeologists suppose that between 670-800 A.D. an Avarian population settled down and lived here. The excavation started in 1985 under the leadership of Medgyesi Pál and was finished in 1989. The total number of individuals found in graves is 243. Among them 64 belongs to the infantia I, infantia II andjuvenis categories in which measures were taken: 15 measurements on the cranium and 17 on the postcranial skeleton taking laterality into account.

As a result of the mathematical-statistical analysis it can be stated that the long bones and the stature grew regularly in all age-groups, and that the mean of the age-groups could be clearly separated which confirms the correct principle of creating them. As expected, comparing the stature of the Avarian children with contemporary individuals shows that contemporary children are taller.

This study is an introductory to a medieval cemetery called Bátmonostor-Pusztafalu, which is the largest medieval cemetery in Hungary. The total number of individuals found in graves and reduction areas is 3783; among them 1510 skeletons belong to the infantia I and infantia II categories. The number ofjuvenis individuals is 153 (Józsa et al. 2004).

Farkas L Gy (2000) Fejezetek abiológiai antropológiából 1. JATE Press, Szeged.
Fazekas L Gy, KósaF (1978) Forensic fetal osteology. Akadémia Kiadó, Budapest.
Józsa L, Farkas L Gy, Paja, L (2004) The frequency of enthesopathies in the 14-15* century series of Bátmonostor-Pusztafalu. Acta Biol Szeged 48(l-4):43-45.
Knussmann R, Martin R (1988) Anthropologie, Handbuch der verglichenden Biologie des Menschen. Gustav Fischer, Stuttgart.
Schour I, Massler M (1941) The development of the human dentition. Journal of the American Dental Assosiation 28:1153-1160.
Visser EP (1988) Little Waifs: Estimating Child Body Size from Historic Skeletal Material. Int J Osteoarchaeology 8:413-423.

Supervisor: Zsuzsanna Just
E-mail: foglagnes@yahoo.co.uk

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The role of FnrT, an oxygen dependent regulator, in the photosynthetic purple sulfur bacterium, *Thiocapsa roseopersicina* BBS

Andrés Fülöp

Department of Biotechnology, University of Szeged, Szeged, Hungary

In facultative anaerobic bacteria the availability of $0_2$is one of the most important regulatory signals (Kovács ÁT et al. 2005a). In the presence or absence of oxygen different metabolic pathways are switched on or off. The FNR protein is an oxygen responsive transcription regulator functioning as a "switch" between the anaerobic and aerobic metabolic pathways. The FNR contains Fe-S clusters which are oxygen sensitive and the *E. coli* FNR was shown to regulate the expression of around 110 operons directly or indirectly (Constantinidou et al. 2006).

Thiocapsa roseopersicina BBS is a Gram-negative, purple sulfur photosynthetic bacterium belonging to the Chromatiaceae family in the y-subdivision of proteobacteria. In addition to the anaerobic photosynthetic growth, the strain is capable to grow aerobically, chemo-lithotrophically in the dark.

My work focuses on the FNR analogue, the FnrT recently identified in *Thiocapsa roseopersicina.*

T. roseopersicina BBS has two sets of membrane-associated [NiFe] hydrogenase genes: the HynSL and HupSL and a third, soluble hydrogenase HoxYH (Kovács KL et al. 2005). In our previous report it was demonstrated that the *hyn* enzyme was anaerobically induced and the upregulation was mediated by the FnrT (Kovács ÁT et al. 2005b). In contrast, *ÜiefnrT*mutation had no effect on the expression of HupSL and HoxYH hydrogenases. Using reporter genes, a slight, negative autoregulation in the expression of the FnrT could be noticed under anaerobic conditions in this bacterium. From these observations an interesting question arose: what is the role of an oxygen sensing protein in a preferentially anaerobic bacterium. Since, the FNR had effect on the expression of more that one hundred genes in *E. coli* we assumed the similar global effect of FnrT in *T. roseopersicina,* as well.

To answer these questions an*fnrT*mutant (FNRTM) strain was prepared. Using proteomic approach, genes, metabolic pathways being under the control of FnrT were/are looked for.

The protein patterns of the wild type and the mutant strain were compared on 2-D gel electrophoresis. On the silver stained gels several spots with distinct intensities were found suggesting a global role *oifnrT* in an - especially anaerobic - photosynthetic sulfur bacterium. In addition, other staining methods are tested to increase the resolution of the protein quantitation, which would allow reliable detection of relatively small differences in the protein level.

Constantinidou C, Hobman JL, Griffiths L, Patel MD, Penn CW, Cole JA, Overton TW (2006) A reassessment of the FNR regulon and transcriptomic analysis of the effects of nitrate, nitrite, NarXL, and NarQP as Escherichia coli K12 adapts from aerobic to anaerobic growth. J Biol Chem 281:4802-4815.

Kovács ÁT, Rákhely G, Balogh J, Maróti G, Fülöp A, Kovács LK (2005a) Anaerobic regulation of hydrogenase transcription in different bacteria. Biochem Soc Trans 33(l):36-38.

Kovács ÁT, Rákhely G, Browing DF, Fülöp A, Maróti G, Busby SJ, Kovács FK (2005b) An FNR-type regulator controls the anaerobic expression of Hyn hydrogenase in Thiocapsaroseopersicina. JBacteriol 187(8):2618-2627.

Kovács FK, Kovács AT, Maróti G, Mészáros FS, Balogh J, Fatinovics D, Fülop A, Dávid R, Dorogházi E, Rákhely G (2005) The hydrogenase of Thiocapsa roseopersicina. Biochem Soc Trans 33(l):61-63.

Fazazzera, BA, Beinert, H, Khoroshilova, N, Kennedy, MC, And Kiley, PJ (1996) DNA binding and dimerization of the Fe-S containing FNR protein from Escherichia coli are regulated by oxygen. J Biol Chem 271:2762-2768.

Supervisors: Gábor Rákhely, Kornél F Kovács
E-mail: fulop@brc.hu

Changes in water relations, stress hormone biosynthesis and glutathione S-transferase activities and expression levels in *Triticum aestivum* cultivars under osmotic stress

Ágnes Gallé

Department of Plant Biology, University of Szeged, Szeged, Hungary

We investigated the drought tolerance of two wheat cultivars *Triticum aestivum* cv. Kobomugi, a near isohydric landrace, and *Triticum aestivum* cv. Ôthalom, a dehydration tolerating genotype. Drought tolerant wheat cultivars exposed to low water potential can be characterized by changes in water relations, production of stress hormones such as abscisic acid (ABA), production of antioxidative enzymes and other signal molecules under osmotic stress. Osmotic stress treatment was applied gradually reaching 400 mOsm polyethylene glycol (PEG 6000) treatment (-0.976 MPa) on one-week-old *Triticum aestivum* plants under controlled conditions as it was published earlier in Erdei (2002). In ABA production, aldehyde oxidase (AO) plays a key role by catalysing the last, rate limiting step of the biosynthesis. After the osmotic stress treatment, the activity of AO increased earlier in the near isohydric cultivar than in the dehydration tolerating Ôthalom. Simultaneously, the cv. Kobomugi accumulated significantly more ABA in the leaf tissue than well-watered control plants, while the ABA

content did not increase in cv. Óthalom. In association with water potential measurements two strategies of acclimation to drought stress have been found. Plants using the first strategy, save tissue water content by a fast decrease of stomatal conductance with fast raise in ABA production and aldehyde oxidase activity. In the second group the closure of stomata occurs later resulting in an intensive loss of water and a fast decrease of water potential in the leaves and tissues restore their turgor after a relatively long acclimation phase. According to our measurements, cv. Kobomugi belongs to the first group, cv. Óthalom into the second group. (Gallé 2002)

Glutathione S-transferase (GST) isoenzymes represents a large and variable group of antioxidative enzymes, with several different activities and sequence patterns. Phylogenetic analysis of wheat *GSTs* was performed *in silico* and using the tentative consensus sequences (TC) a dendogram was composed. According to the conserved sequences used for classification of GST proteins, we could identify four groups of wheat GSTs *(phi, zeta, theta* and *tau)*. Homology was found between the osmotic stress upregulated sequences and the GST coding TCs were identified. Real Time PCR analysis with two group-specific primer showed a significant increase in the amount of GST transcripts after plants were exposed to 400 mOsm PEG treatment for two days (Gallé 2005). The changes in the transcripts were compared with the GST activities measuring in the same times. According to our results the members of *phi* GST can be responsible for the fast response after the osmotic stress, while the *tau* GST group for the later enhancement of GST activity.

Gallé Á, Csiszár J, Secenji M, Tari I, Gyorgyey J, Dudits D, Erdei L (2005) Changes of glutathione S-transferase activities and gene expression in Triticum aestivum during polyethylene-glycol induced osmotic stress. Acta Biol Szeged 49:95-96.
Gallé Á, Csiszár J, Tari I, Erdei L (2002) Changes in water and chlorophyll fluorescence parameters under osmotic stress in wheat cultivars. Acta Biol Szeged 46: 85-86.

Supervisor: Jolán Csiszár
E-mail: gallea@bio.u-szeged.hu

Identification and characterization of *Trichoderma* strains causing mushroom green mould disease in Hungary

Lóránt Hatvani

Department of Microbiology, University of Szeged, Szeged, Hungary

World-wide mushroom cultivation is dominated by the production of *Agaricus bisporus, Lentinula edodes* and *Pleurotus ostreatus* (Chang 1999). Producers of champignon (Agaricus bisporus) and oyster mushroom *(Pleurotus ostreatus)* are facing recent incidents of green mould epidemics in Hungary.

We examined 66 *Trichoderma* strains isolated from *Agaricus* compost and *Pleurotus* substrate samples from three Hungarian mushroom producing companies by a PCR-based, diagnostic test for *T. aggressivum* (Chen et al. 1999), sequence analysis of the internal transcribed spacer regions 1 and 2 (Druzhinina et al. 2005) and (selectively) of the 4th and 5th intron of translation elongation factor la (4), and RFLP of mitochondrial DNA.

Seven *Trichoderma* species were identified [numbers of isolates given in brackets]: *T. aggressivum f. europaeum* [17], *T. harzianum* [3], *T. longibrachiatum* [4], *T. ghanense* [1], *T. asperellum* [4], *T. atroviride* [9], and a still undescribed phylogenetic species: *Trichoderma* sp. DAOM 175924 [28]. *T. aggressivum f. europaeum* was exclusively derived from A. bisporus compost, whereas *Trichoderma* sp. DAOM 175924 exclusively occurred in the substrate for *Pleurotus* cultivation. Sequences of the latter strains were cospecific with those for *Trichoderma* pathogens of *P. ostreatus* in Korea. The isolates of T. sp. DAOM 175924 can be divided into two types on the basis of an A/C transversion at position 447 in ITS2. The two types of *T.* sp. DAOM 175924 isolates differ from each other based on morphological features as well, and they are currently being described as new species, *T. fulvidum* sp. nov. and *T. pleurotophilum* sp. nov., respectively.

The widespread occurence of this new species raises the questions, why infections by it havejust only recently been observed. Our data document that (a) green mould disease by *T. aggressivum f. europaeum* has geographically expanded to Central Europe; (b) the green mould disease of *P. ostreatus* in Hungary is due to the same *Trichoderma* species as in Korea and the world-wide distribution of the new species indicates the possibility of spreading epidemics; and (c) on mushroom farms, the two species are specialized on their different substrates.

Chang ST (1999) World production of cultivated and medicinal mushrooms in 1997 with emphasis on Lentinus edodes (Berk.) Sing, in China. Int J Med Mushrooms 1:291-300.
Chen X, Romaine CP, Ospina-Giraldo MD, Royse DJ (1999) A polymerase chain reaction-based test for the identification of Trichoderma harzianum biotypes 2 and 4, responsible for the worldwide green mold epidemic in cultivated Agaricus bisporus. Appl Microbiol Biotechnol 52:246-250.
Druzhinina I, Kopchinskiy AG, Komon M, Bissett J, Szakács G, Kubicek CP (2005) An oligonucleotide barcode for species identification in Trichoderma and Hypocrea. Fungal Genet Biol 42:813-828.
Kopchinskiy A, Komon M, Kubicek CP, Druzhinina I (2005) TrichoBLAST: a multilocus database for Trichoderma and Hypocrea identifications. Mycol Res 109:657-660.

Supervisor: László Manczinger
E-mail: lori@szegedkendo.hu

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Study of experimental inflammatory bowel disease in the rat

Krisztina Horváth

Department of Comparative Physiology, University of Szeged, Szeged, Hungary

The pathogenesis of the inflammatory bowel diseases is still incompletely understood. It is likely that local release of reactive oxygen species (ROS) may be involved, creating epithelial injury in the colon (Simmonds et al. 1993; McKenzie et al. 1996; Dryden et al. 2005). It is therefore possible that endogenous protective antioxidant systems could be evoked in order to attenuate colonic tissue injury. ROS may be modulated by endogenous anti-oxidant products of heme oxygenase-1 (HO-1). In the present work, HO-1 expression in trinitrobenzene sulphonic acid (TNBS)-induced colitis in the rat and the effects of HO-1 modulation, particularly by the HO-1 inducer, heme, were further evaluated. Colitis was induced by intracolonic challenge with TNBS and assessed macroscopically and by myeloperoxidase (MPO) assay. TNBS challenge led to an early and substantial induction of HO-1 protein expression and HO activity in the colon that peaked after 48-72 h and declined over 10 days. Heme (30 pmol/kg/day, s.c) increased colonic HO-1 protein expression and HO enzyme activity and decreased colonic damage and MPO activity. In contrast, the HO-1 inhibitor, zinc protoporphyrin (ZnPP; 50 pmol/kg/day, s.c) significantly increased the colonic damage and MPO activity over 10 days, as did tin protoporphyrin (30 pmol/kg/day, s.c). These results support the proposal that induction of HO-1 provides a protective mechanism in this model under both acute and more-chronic conditions, and that its selective up-regulation could thus be of therapeutic potential in colitis.

The mechanism of action of 5-aminosalicylic acid (5-ASA), the active therapeutic moiety of a number of clinically used anti-colitic agents, has long been considered to include its direct anti-oxidant and radical scavenging activity. The present study also investigates whether this effect *in vivo* could also involve induction of HO-1, known to provide endogenous anti-oxidant and anti-inflammatory moieties which can modulate colonic inflammation. However, an enduring concept is that at least part of the beneficial activity of 5-ASA reflects its actions as an antioxidant and free radical scavenger (Simmonds et al. 1999; Reifen et al. 2004).

The effects of 5-ASA on the colonic expression and activity of HO-1 along with its effect on the inflammatory damage have been evaluated in the colitis provoked by instillation of TNBS over 48 hours. Intra-colonic administration of 5-ASA (8, 25 and 75 mg kg'day^{1}) dose-dependently reduced the TNBS-provoked macroscopic colonic inflammatory injury and MPO levels, while also dose-dependently increasing colonic heme oxygenase enzyme activity (by 42%, 46% and 77% respectively at the highest dose). Colonic HO-1 activity expression was likewise further induced by 5-ASA. Moreover, intra-colonic administration of 5-ASA alone under unchallenged conditions induced colonic HO-1 expression and stimulated HO activity. Administration of ZnPP (30 pmol/kg/day, s.c.), which prevented the increase in colonic HO activity, abolished the anti-colitic effect of 5-ASA.

These results suggest that 5-ASA may exert its colonic anti-oxidant and anti-inflammatory effects *in vivo* in part through the up-regulation of HO-1 enzyme expression and HO activity.

Dryden Jr GW, Deaciuc I, Arteel G, McClain CJ (2005) Clinical implications of oxidative stress and antioxidant therapy. Curr Gastroenterol Rep 7:308-316.
Grisham MB, Volkmer C, Tso P, Yamada T (1991) Metabolism of trinitrobenzene sulfonic acid by the rat colon produces reactive oxygen species. Gastroenterology 101:540-547.
McKenzie SJ, Baker MS, Buffinton GD, Doe WF (1996) Evidence of oxidant-induced injury to epithelial cells during inflammatory bowel disease. J Clin Invest 98: 136-141.
Reifen R, Nissenkom A, Matas Z, Bujanover Y (2004) 5-ASA and lycopene decrease the oxidative stress and inflammation induced by iron in rats with colitis. J Gastroenterol 39:514-519.
Simmonds NJ, Millar AD, Blake DR, RamptonDS (1999) Antioxidant effects of aminosalicylates and potential new drugs for inflammatory bowel disease: assessment in cell-free systems and inflamed human colorectal biopsies. Aliment Pharmacol Ther 13:363-372.
Simmonds NJ, Rampton DS (1993) Inflammatory bowel disease - a radical view. Gut 34:865-868.

Supervisor: Csaba Varga
E-mail: hkriszta@bio.u-szeged.hu

Analysis and identification of pathogen *Candida* species based on molecular variability and DNA sequences

Sándor Kocsubé

Department of Microbiology, University of Szeged, Szeged, Hungary

Candida species are members of the normal human flora and can be easily recovered from our environment. Infections caused by *Candida* species are widespread throughout the world. Although *Candida albicans* is the most common *Candida* species encountered as a cause of human infections, other *Candida* species have been increasingly associated with disseminated disease since the 1990s. Among them, *Candida parapsilosis* has been the second most common yeast species isolated from bloodstream infections in several surveys (Messer et al. 2006). This species has emerged as an important nosocomial pathogen, with clinical manifestations including fungemia, endocarditis, endophthalmitis, septic arthritis, and peritonitis, usually occur in association with invasive procedures or prosthetic devices This species is

more frequent in bloodstream infections of neonates, in transplant recipients, and in patients who received parenteral nutrition or previous antifungal therapy.

There is no completely reliable method for detecting *Candida* infections. Although there are good methods for identifying *Candida* species, they are often time- and cost-consuming. Our aim was to develop a rapid and reliable molecular method to identify clinically important *Candida* species from bloodstream infections, and to examine the genetic variability of *Candidaparapsilosis*.

For the development of the *Candida* specific primer pairs, we used DNA sequences of interest avaiable in the National Center for Biotechnology Information (NCBI) database. Missing sequences were supplied by direct sequencing of the adequate fragments. Eight primer pairs were developed, capable of specifically identifying *Candida glabrata, C. guilliermondii, C. krusei, C. lusitaniae, C. albicans, C. dubliniensis, C. tropicalis* and *C.parapsilosis.*

To analyse the genetic variability of *Candida parapsilosis* isolates, 209 *Candida* isolates from blood samples from two Hungarian hospitals located in Debrecen and Pécs were examined. The samples came from different patients and identified by standard morphological and physiological methods within the hospitals.

Previous studies clarified that *C. parapsilosis* isolates can be divided into three groups which could be distinguished based on several criteria including randomly amplified polymorphic DNA (RAPD) analysis, sequences of the internal transcribed spacer (ITS) region of the rRNA gene cluster, DNA relatedness, morphotyping, electrophoretic karyotypes, single nucleotide polymorphisms, mitochondrial DNA sequence differences and biofilm producing abilities. Recently, Tavanti et al. (2005) recognized *C. parapsilosis* groups II and III as separate species, *C. orthopsilosis* and *C. metapsilosis,* respectively, based on multilocus sequence typing studies. The two latter species can be recovered relatively rarely in clinical samples. Besides, *C. parapsilosis* group IV has also been found recently among Brazilian clinical *Candida* isolates by Iida et al. (2005). We examined the occurrence of *C. parapsilosis* isolates among *Candida* isolates collected in Hungarian hospitals, and examined the genetic variability of these isolates using sequence analysis of the ITS region, and RAPD technique. Two isolates were found to belong to the recently described *C. metapsilosis* species (C. *parapsilosis* group 3) based on molecular and phenotypic data. This is the first report on the identification of *C. metapsilosis* from bloodstream infection.

Iida S, Imai T, Oguri T, Okuzumi K, Yamanka A, Moretti-Branchini M, Nishimura K, Mikami Y (2005) Genetic diversity of the internal transcribed spacers (ITS) and 5.8S rRNA genes among clinical isolates of Candida parapsilosis in Brazil and Japan. Jpn J Med Mycol 46:133-137.

Messer SA, Jones RN, Fritsche TR (2006) International surveillance of Candida spp. and Aspergillus spp. Report from the SENTRY Antimicrobial Surveillance Program (2003). J Clin Microbiol 44:1782-1787.

Tavanti A, Davidson AD, Gow NA, Maiden MC, Odds FC (2005) Candida orthopsilosis and Candida metapsilosis spp. nov. to replace Candida parapsilosis groups II and III. J Clin Microbiol 43:284-292.

Supervisor: János Varga
E-mail: kocsube@invitel.hu

The physiological role of a NAD^+-reducing cytoplasmic hydrogenase in a phototrophic bacterium, *Thiocapsa roseopersicina* BBS

Dóra Latinovics

Department of Biotechnology, University of Szeged, Szeged, Hungary

In the near future, the supply of fossil fuels will be likely exhausted and other alternative energy sources and carriers have to be found. Hydrogen seems to be one of the best energy carriers, since its burning leads to water, therefore a hydrogen economy would substantially contribute to the reduction of $C0_2$ emission and - consequently - global warming. Biological systems, as renewable catalysts, are ideal tools for production of hydrogen, named as biohydrogen. There are few ways for production of biohydrogen, and - in most cases - the key enzyme in the process is the hydrogenase.

Our model organism, *Thiocapsa roseopersicina* BBS is a phototrophic purple sulfur bacterium, which harbours several [NiFe] hydrogenases. The HynSL and HupSL hydrogenases are associated to the membrane, while the HoxEFUYH, a NAD^+-reducing enzyme - being responsible for the light dependent H_2 evolution of the cells - could be identified in the soluble fraction. The *hox* gene products showed the highest similarity to the corresponding subunits of the cyanobacterial bidirectional hydrogenases (HoxEFUYH), which form a separate subfamily of the NAD^+ reducing hydrogenases [1].

There is an obvious question to be answered: why a single cell needs so many isoenzymes, what are their physiological roles. Here, I focused on the physiological characterization of the *hox* genes and enzymes.

Downstream from the *hoxH* gene the *hoxW* gene was indentified, which was likely responsible for the posttranslational cleavage of the HoxH subunit. Reverse transcription coupled PCR experiments revealed that all five structural genes together with the *hoxW* gene were localized on a single transcript. This indicated concerted regulation of the gene cluster.

Very little is known on the the expression pattern of the Hox enzyme. So far, environmental factor effecting its expression level could not be identified. However, using the *lacZ* reporter gene fused to a deletion series of the upstream regulatory region of the *hox* operon, a section having negative impact on the expression of the *hox* gene cluster could be perceived. The fine mapping of this *cis* regulatory element and identification of the corresponding transcription factor(s) are in progress.

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The presence of the HoxE subunit is exclusively characteristic for this subfamily of the NAD^+-reducing [NiFe] hydrogenases. *In frame* deletion of the *hoxE* gene abolished the hydrogen evolution derived from the Hox enzyme *in vivo,* although it had no effect on the activity *in vitro.* This suggested, that HoxE had a hydrogenase-related role: probably it took part in the electron transfer processes.

With the aid of a C-terminal double-tagged construct [2] we could purify the HoxE protein together with the HoxFUYH subunits of the Hox hydrogenase. This confirmed the fact that the HoxE protein is part of the multisubunit hydrogenase-complex. Similar approach is used to identify electron donor/acceptor proteins interacting with the HoxE subunit and linking the Hox enzyme to the respiratory and/or photosynthetic complexes.

Fodor BD, Kovács AŤ, Csáki R, Hunyadi-Gulyás E, Élement E, Ḿaróti G, Mészáros LS, Medzihradszky KF, Rákhely G, Kovács KL (2004) Modular broad-host-range expression vectors for the purification of proteins and protein complexes and their application. Appl Environ Microbiol 70(2):712-721.

Rákhely G, Kovács AŤ, Maróti G, Fodor BD, Csanádi Gy, Latinovics D, Kovács KL (2004) Cyanobacterial-type, heteropentameric, NAD+-reducing NiFe hydrogenase in the purple sulfur photosynthetic bacterium Thiocapsa roseopersicina. Appl Environ Microbiol 70(2):722-728.

Supervisors: Gábor Rákhely, Kornél Kovács
E-mail: ldora@szbk.hu

Effect of cell density on lipid composition and heat shock protein response

Andriy Maslyanko

Institute of Biochemistry, Biological Research Centre, Hungarian Academy of Sciences, Szeged, Hungary

Life requires the presence of a barrier around cells, but the cell membrane is not merely a barrier that must be traversed; rather, the membrane and its constituent lipids are also indispensable participants in many events of signal transduction. The field of signal transduction is important because of its fundamental role in cellular communication and regulation of cellular responses to various stresses (Kathleen M. 2007). It was suggested, that the extent of membrane damage and cellular tolerance limits during heat stress depends on lipid composition, fatty acid saturation and membrane fluidity/microdomain organization of the cell membrane (Horváth et al. 1998; Vigh et al. 1998; Vígh and Maresca 2002; Vigh et al. 2005).

When cells are exposed to temperatures that exceed their normal growth temperature, they respond by inducing the synthesis of several polypeptides referred to as heat shock proteins (HSPs). We have demonstrated, that subtle membrane perturbations are critically involved in the conversion of signals from the environment into the transcriptional activation of various hsp genes (Nagy et al. 2007). A specific set of HSPs, like HSP25 and HSP70 are expressed in response to a wide variety of physiological and environmental insults, thus allowing the cells to survive lethal conditions. Several mechanisms account for the cytoprotective effect of HSP25 and HSP70. Both proteins are powerful molecular chaperones. They both inhibit key effectors of the apoptotic machinery at the pre- and post-mitochondrial level and participate in the proteasome-mediated degradation of proteins under stress conditions.

In cancer cells, the expression of HSP25 and/or HSP70 is typically abnormally high, and both HSPs may participate in oncogenesis and in resistance to chemotherapy. In rodent models, overexpression of HSP25 and/or HSP70 increases tumor growth and metastatic potential. By contrary, silencing the hsp25 gene eliminates completely the migration capability of certain highly metastatic tumor cells. In addition, it was recently shown, that cellular distribution and especially surface membrane expression of HSP25 and HSP70 differentially regulates tumor growth and metastasis (Bausero et al. 2004). Therefore, understanding the key factors contributing to the control of the expression and cellular localization of these HSPs has become a novel strategy of cancer therapy.

In our study we investigated the effect of different plating densities on the membrane fatty acid (FA) composition of B16-F10 melanoma cells, an established cellular model of melanoma. We have shown that small variations in the initial cell density could profoundly influence the FA composition of membrane lipids. With increasing cell number we observed a strong enrichment of oleic acid content paralleled with the decline of the level of specific polyene fatty acids (mainly the arachidonic acid). Even the saturated FA-s were lessened presumably by being substrates of delta-9 desaturase. Changes in cholesterol level and the accumulation of desmosterol, a percursor of cholesterol were also detected. We could conclude, that cell density is a major determinant of lipid composition of our model system.

In B16-F10 cells the elevated mRNA product for HSP25 and HSP70 were reliably observed at 41°C and strongly increased upon exposure to 42°C. We have shown, that parallel with causing different lipid composition variation of initial cell density profoundly altered both the amplitude and ratio of mRNA levels corresponding to HSP25 and HSP70 under mild, physiologically relevant heat stress conditions. Suprisingly, the synthesis of these major heat shock proteins does not necessarily correlate with mRNA levels. Based on these results we could hypothesize that transcription and translation processes are not directly coupled events under conditions investigated.

Taken together, our studies first identify an important role of culture conditions and especially the initial cell densities for controlling the expression of HSP25 and HSP70 during mild heat stress conditions which might be helpful in the future design of antitumor therapies.

Bausero MA, Page DT, Osinaga E, Asea A (2004) Surface expression of Hsp25 and Hsp72 differentially regulates tumor growth and metastasis. Tumour Biol 25(5-6):243-251.

Horváth I, Glatz A, Varvasovszki V, Török Z, Pali T, Balogh G, Kovács E, Nadasdi L, Benko S, Joo F, Vigh L (1998) Membrane physical state controls the signaling mechanism of the heat shock response in Synechocystis PCC 6803: Identification ofhspl7 as a "fluidity gene". Proc Natl Acad Sci USA 95:3513-3518.

Kathleen M (2007) The membrane and lipids as integral participants in signal transduction: lipid signal transduction for the non-lipid biochemist. Adv Physiol Educ

31:5-16.
Nagy E, Balogi Zs, Gombos I, Akerfelt M, Björkbom A, Balogh G, Török Zs, Maslyanko A, Lisowska K, Slotte P, Sistonen L, Horváth I, Vígh L (2007) Hyperfluidization coupled membrane microdomain reorganization is linked to the activation of the heat shock protein response in murine melanoma cell line. Proc Natl Acad Sci USA (in press).
Vígh L, Maresca B, Harwood J (1998) Does the membrane's physical state control the expression ofheat shock and other genes? Trends Biochem Sci 23:369-374.
Vígh L, Maresca B (2002) Dual role of membrane in heat stress: As termosensor modulate the expression of stress genes and, by interacting with stress proteins, reorganize their own lipid order and functionality. Cell and Molecular Responces to Stresses, Eds. KB Storey and JM Storey, Elsevier Science, pp. 173-188.
Vígh L, Escrima PV, Sonnleitner A, Sonnleitner M, Piotto S, Maresca B, Horváth I, Harwood JL (2005) The significance of lipid composition for membrane activity: New concepts and ways of assensing function. Prog Lipid Res 44:303-344.

Supervisor: László Vígh
E-mail: andriy@brc.hu

Toxin - antitoxin modules affects the stress response and metabolism in Rhizobia

Sebastian Paul Miclea

Institute of Genetics, Biological Research Center, Hungarian Academy of Sciences, Szeged, Hungary

The recent expansion of microbial DNA and protein databases that followed the sequencing of a large number of prokaryotic genomes has promoted the identification of numerous toxin-antitoxin modules present in the bacterial plasmids and chromosomes. Chromosomal TA modules are characterized by conserved structural organization, transcriptional autoregulation and a similar function, although their targets in cells are different (2). The two adjacent genes of the operon partly overlap at the stop/start codons. The two proteins form a complex, thus the antitoxin prevents the lethal or bacteriostatic effect of the toxin (3, 5). The generally accepted idea about the function of these chromosomally located systems is that they act as bacterial metabolic stress managers, being associated with the modulation of the global level of translation under conditions of nutrient limitation, or under stress conditions (2).

Rhizobia are soil bacteria capable of eliciting specialized root organs, known as nodules on the roots of leguminous plants, in which they reduce dinitrogen. In this unique association between eukaryotes and prokaryotes, the plant provides the source of energy for the bacterium, which in return synthesizes ammonia for the host plant. Rhizobia as symbiotic bacteria have to face various stresses: different environmental conditions in the soil and inside the root nodule and different nutrition circumstances in free-living and symbiotic state. The TA systems may be involved in helping these bacteria to cope with these transitions. The previously identified *ntrPR* operon in *Sinorhizobium meliloti,* the microsymbiont of alfalfa, represents such a chromosomally located TA module, where the *ntrP* is the antitoxin and the *ntrR* is the toxin component of the module (2). As was demonstrated earlier, a Tn5 insertion in the *ntrR* gene resulted in increased nodulation and more efficient nitrogen fixation capacity (6). When the gene expression patterns of the entire genomes of the wild type and *ntrR* mutant strains were compared, an unexpectedly large number of genes exhibited altered expression in the mutant strain, suggesting a general modulating function for NtrR (7). Among the genes with altered expression, genes coding for several chaperones were identified. Chaperones play an important role in minimizing the cellular damage caused by stress conditions such as heat shock, infection, oxygen deprivation, exposure to high salt (4). A multigene family of chaperonins was identified in *S. meliloti (groESLl, groESL2, groESL3, groEL4, groEES).*

To determine the involvement of chaperonines in the protection of cells from the stress-induced damage, wild type and *ntrR* mutant bacteria containing a *groEL5::lacZ* translational fusion were grown at high temperature and at high salt concentrations. The *groEL5 - lacZ* fusion measurements revealed that the expression of this stress related gene is under the control of the *ntrR* gene and the signals for induction of GroEL5 synthesis involve high temperatures and oxygen limitation. An apparent "salt - tolerance" of *groEL5* in the *ntrR* mutant was also determined.

Using a biocomputational approach we also identified a TA like module in *Bradyrhizobiumjaponicum,* the microsymbiont of soybean, in which one gene of the operon shows a high degree of homology at the level of aminoacid sequence with the *ntrR* toxin of *S. meliloti.* The chromosomal operon bsl2435/bll2434 (designated as *bat/bto* operon) of the symbiotic bacterium *B.japonicum* encodes a protein pair, which forms a possible toxin-antitoxin module. In order to determine the functional role of this module we constructed a *B.japonicum* mutant in which the whole *bat/bto* operon was deleted. The effect of this mutation was a boost of the growth of these bacteria. *B.japonicum* belongs to the so-called slow growing rhizobia with a doubling time of approximately 14 hours compared to a doubling time of 3-4 hours of *S. meliloti.* The deletion mutant constructed by us has a faster metabolic rate with a doubling time of 1.5-2.5 hours depending on the composition of the media.

In order to develop symbiotically more efficient and stress tolerant strains, the involvement of toxin - antitoxin modules in stress response and metabolism of bacteria belonging to Rhizobiaceae is under further investigation.

Bodogai M, Ferenczi S, Bashtovyy D, Miclea P, Papp P, Dusha I (2006) The ntrPR operon of Sinorhizobium meliloti is organized and functions as a toxin-antitoxin module. Mol Plant Mirobe Interact 19:811-822.
Buts L, Lah J, Dao-Thi MH, Wyns L, Loris R (2005) Toxin-antitoxin modules as bacterial metabolic stress managers. Trends Biochem Sci 30:672-679.
Engelberg-Kulka H, Glaser G (1999) Addiction modules and programmed cell death and antideath in bacterial cultures. Annu Rev Microbiol 53:43-70.
Fischer HM, Babst M, Kaspar T, Acuña G, Arigoni F, Hennecke H (1993) One member of a gro-ESL-like chaperonin multigene family in Bradyrhizobium japonicum is co-regulated with symbiotic nitrogen fixation genes. EMBO J 12:2901-2912.

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Acta Biologica Szegediensis
http://www.sci.u-szeged.hu/ABS

Gerdes K (2000) Toxin-antitoxin modules may regulate synthesis of macromolecules during nutritional stress. J Bacteriol 182:561-572.
Oláh B, Kiss E, Györgypál Z, Borzi J, Cinege G, Csanádi G, Batut J, Kondorosi A, Dusha I (2001) Mutation in the ntrR gene, a member of the vap gene family, increases the symbiotic efficiency of Sinorhizobium meliloti. Mol Plant-Microbe Interact 14:887-894.
Puskás LG, Nagy ZB, Kelemen JZ, Rüberg S, Bodogai M, Becker A, Dusha I (2004) Wide-range transcriptional modulating effect of ntrR under microaerobiosis in Sinorhizobium meliloti. Mol Gen Genomics 272:275-289.

Supervisor: Ilona Dusha
E-mail: Micleasp@nucleus.szbk.u-szeged.hu

Analysis of the transcriptional regulation of the matrilin-1 gene in transgenic mice

Andrea Nagy

Institute of Biochemistry, Biological Research Center, Hungarian Academy of Sciences, Szeged, Hungary

Matrilin-1 is a non-collagenous protein which functions in the organization of the extracellular matrix. It has the unique feature among cartilage-specific genes that its expression is restricted to distinct zones of the growth plate *in vivo*. In tissue cultures it also shows developmental stage-specific expression. Previous analysis of the matrilin-1 control regions in transgenic mice revealed that the presence of both promoter upstream and intronic elements was necessary for the high-level transgene activity in all chondrogenic tissues and for the extraskeletal transgene expression pattern resembling the most to that of the chicken matrilin-1 gene. We also found that the long promoter alone and the short promoter in combination with the intronic enhancer restricted the transgene expression to the zones of proliferative chondroblasts and prehypertrophic chondrocytes in the growth-plate cartilage (Karcagi et al. 2004). Since these transgenes shared only the short promoter, we raised the question whether the short promoter was responsible for the zonal and tissue-specific expression pattern. Based on computer analysis conserved sequence blocks were found within this region. Previous experiments also showed that these elements can bind to a cartilage-specific transcription factor (Rentsendorj et al. 2005) which plays key role in chondrogenesis. To test the contribution of these elements to the regulation of the gene, we generated *luciferase* and *LacZ* fusion constructs with the short promoter - or its mutant forms - and different combinations of the distal promoter elements. Apart from matrilin-1 regulatory regions, we also tested the promoter activity under the control of heterologous regulatory elements. Activity of the luciferase fusion constructs was measured in transient expression assays in different cell cultures. High density mesenchyme and chicken embryonic chondrocyte consisted mostly of early proliferative and late proliferative chondrocytes, respectively, whereas chicken embryonic fibroblast represented the non-expressing cell type. Those combinations of regulatory regions which showed appropriate activity in transient expression assays, were inserted into a *LacZ* reporter containing plasmid. These constructs were microinjected into the male pronuclei of fertilized mouse eggs. The transgene expression was monitored by whole-mount X-gal staining and histological analysis in the growth plate of GO founder embryos. We found that the short promoter alone exhibited relatively low activity with preference to zones of proliferating chondroblasts and prehypertrophic chodrocytes of the growth plate (Rentsendorj et al. 2005). Insertion of upstream elements to the short promoter raised the activity of the transgenes. Analysis of the effect of homologous and heterologous enhancer elements and the mutant versions provided further insight into the transcriptional regulation of the matrilin-1 gene.

Karcagi I, Rauch T, Hiripi L, Rentsendorj O, Nagy A, Bősze Zs, Kiss I (2004) Functional analysis of the regulatory regions of the matrilin-1 gene in transgenic mice reveals modular arrangement of tissue-specific control elements. Matrix Biol 22:605-618.
Rentsendorj O, Nagy A, Sinkó I, Daraba A, Barta E, Kiss I (2005) Highly conserved proximal promoter element harbouring paired Sox9-binding sites contributes to the tissue- and developmental stage-specific activity of the matrilin-1 gene. Biochem J 389:705-716.

Supervisor: Ibolya Kiss
E-mail: nagya@brc.hu

Systems biology analysis of a novel microfibrillar network associated human fibrotic disorder

Anita Ordas

Department of Genetics, University of Szeged, Szeged, Hungary, Cardiovascular Research Center, University of Hawaii, Honolulu, HI, USA, Hospital for Sick Children, Toronto, Canada

A female patient was diagnosed with a novel connective tissue disorder at the age of 5. Her symptoms, now at her age of 19, include severe, progressive contractures of the leg and handjoints, areas of her skin appear fragile, thin, and translucent with fibrotic papulae, that resemble hypertrophic scars. These papulae along with collagenous bundles are prevalent at areas of skin under tension from growth orjoint flexion.

Histopathology of a skin biopsy showed normal epidermis, preserved skin appendages, and extensive fibrosis within the dermis characterized by dense hypocellular bands and nodules of collagen. Ultrastructural analysis of a skin biopsy revealed a slight variation in the size of the collagen fibers; in addition to normal, 50 nm diameter fibers, abnormally small, 32 nm fibers were also detected. The genetic cause of the disorder is unknown.

The aim of our study was to identify the molecular background of this disorder. Increased collagen fiber accumulation suggested lysyl oxidase (LOX) involvement. LOX is an amine oxidase that plays an essential role in the catalysis of lysine-derived crosslinks in extracellular matrix (ECM) proteins including collagens and elastin (Kagan and Li 2003). We, indeed, detected elevated LOX, and LOX-like (LOXL) mRNA, protein, and catalytic activity levels in dermal fibroblast cultures derived from the patient's skin biopsy.

We performed whole genome expression array analysis that revealed that in addition to *LOX* and *LOXL,* other important ECM genes were also differentially expressed. Notably, the majority of genes encoding microfibrillar network associated proteins, including fibrillin-2 *(FBN2),* microfibril associated glycoprotein-2 (*MAGP2*), fibulin-1 and -3 *(FBLN1,3)* were significantly downregulated. Microfibrillar network is the template and regulator of collagen and elastin fibrillogenesis. Immunofluorescent staining of fibrillin-1 (LBN1) and elastin demonstrated that altered microfibrillar network in the patient's cultured dermal fibroblasts resulted in disorganized collagen and elastin fiber assembly that might contribute to the development of the severe dermal phenotype in the patient.

The patient's clinical symptoms have overlapping features with Marfan syndrome, Congenital Contractural Arachnodactyly (CCA) and scleroderma. Mutational analysis of *FBN1, FBN2* (genes possessing mutations causing Marfan syndrome and CCA, respectively), and *MAGP1, MAGP2* (genes whose products interact with LBN1, and LBN2) did not detect any mutations. Therefore, Marfan syndrome and CCA can probably be excluded as a causative for this phenotype. In contrast, the comparison of the transcriptional profile of our patient to the transcription profile of scleroderma patients identified partial overlap in differentially expressed ECM genes (9 out of 18; Tan et al. 2005). However, the mechanism of fibrosis is most probably different in this patient, since in fibrotic disorders, including scleroderma, TGF-p plays an important role in the development of the condition and it causes the upregulation of *lox* mRNA, but in our case microarray analysis and qRT-PCR results revealed that no TGF-p genes, TGF-p receptors or ECM genes known to be regulated by TGF-p were upregulated (Denton and Abraham 2001).

These data suggest that this fibrotic disorder might be a new type of severe, early onset scleroderma or a yet uncharacterized microfibrillar network associated fibrotic disorder, where elevated LOX and decreased microfibrillar protein levels are regulated independently of the TGF-p pathway.

Denton CP, Abraham DJ (2001) Transforming growth factor-beta and connective tissue growth factor: key cytokines in scleroderma pathogenesis. Curr Opin Rheumatol 13(6):505-511.

Kagan HM, Li W (2003) Lysyl oxidase: properties, specificity, and biological roles inside and outside of the cell. J Cell Biochem 88(4):660-672.

Tan FK, Hildebrand BA, Lester MS (2005) Classification analysis of the transcriptosome of nonlesional cultured dermal fibroblasts from systemic sclerosis patients

Supervisors: Katalin Csiszár and Mátyás Mink
E-mail: ordasanita@yahoo.com

Identification and characterization of genes involved in abiotic stress responses in *Arabidopsis*

Csaba Papdi

Institute of Plant Biology, Biological Research Center, Hungarian Academy of Sciences, Szeged, Hungary

Land plants struggle with adverse environmental factors such as drought, salinity, and cold during their lifetimes (Bohnert et al. 1995). In response to these factors changes occur in physiological and molecular level controlled by cell signaling networks (Xiong et al. 2002).

The major plant stress hormone abscisic acid has a complex regulation. A number of genes have been indentified as positive or negative regulators of ABA signaling (Finkelstein and Rock 2002). One of the most important aspect of understanding plant stress regulation is elucidation of ABA dependent and independent processes.

To indentify *trans*-acting regulators of the well characterized ABA inducible gene *alcohol dehydrogenase 1 (adhl)* we have constructed luciferase reporter gene fusions with the promoter *oiADHl* gene, and tested the applicability in genetic screen experiments.

We have developed a new genetic technology to identify yet unknown stress related factors. A full-length cDNA expression library has been constructed using RNA templates isolated from different tissues of *Arabidopsis* seedlings under salt stress conditions. It has been cloned into pER8 - an estradiol inducible plant expression vector (Zuo et al. 2000), and transformed into *Arabidopsis* plants that contain the *ADHl-luc* reporter construct. We have indentified an *Arabidopsis* transformant line, in which estradiol induction of cDNA expression frans-activates the *Arabidopsis ADH1* promoter in the absence of stress signals. We determined that cDNA is a transcripton factor Related to Apetala 2.12 (RAP2.12) belongs to the AP2/EREBP gene family. In further experiments we revealed that the induced RAP2.12 expression and the hormone ABA have a synergistic effects on *ADH1* promoter. The cDNA of RAP2.12 has been re-cloned into the estradiol inducible and constitutive expression vector and retransformed into the tester *Arabidopsis* line to confirm its functional effects.

Bohnert HJ, Nelson DE, Jensen RG (1995) Adaptation to environmental stresses. Plant Cell 7:1099.

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http://www.sci.u-szeged.hu/ABS

Xiong L, Schumaker KS, Zhu JK (2002) Cell signaling during cold, drought, and salt stress. Plant Cell Suppl:S165.
Finkelstein RR, Rock CD (2002) Abscisic acid biosynthesis and response. The Arabidopsis book, American Society of Plant Biologists, Rockville, MD doi: 10.1199/tab.0009, www.aspb.org/publications/arabidopsis/
Zuo J, Niu QW, Chua NH (2000) Technical advence: An estrogen receptor-based transactivator XVE mediates highly inducible gene expression in transgenic plants. Plant J 24:265.

Supervisor: László Szabados
E-mail: papdi@brc.hu

Drosophila p53- a model for the better understanding of human p53 function

Norbert Pardi

Department of Biochemistry and Molecular Biology, University of Szeged, Szeged, Hungary

The p53 protein plays a vital role in safeguarding the integrity of the genome by inhibiting the cell cycle, or bringing about apoptosis in case of different cellular stresses. In accordance with this role mutations of the tumor suppressor genepJi are frequently found in different types of cancer. Identification of the *Drosophila melanogaster* homologue of p53 (Dmp53) opens up possibilities to apply a combination of biochemical and genetical tools for studying p53 functions in flies.

We analysed several interacting partners of the Dmp53 and determined the interacting regions of the protein using the yeast two-hybrid assay. Two main aspects of this work is reported here.

Several of the identified Dmp53-interacting proteins (dUba2, lwr, dPias) are involved in the process of sumoylation. Sumoylation is a posttranslational modification in which a small polypeptide is attached to a target protein resulting in a great variety of changes in the life of the modified protein (changes in nuclear transport, transcriptional activity etc...). The human p53 has been shown to be sumoylated. We found a putative sumoylation site at the C-terminus of the Dmp53 protein as well. Taken together, we suggest that the Dmp53 protein, like its human counterpart, can be sumoylated. Using the yeast two-hybrid assay we characterized the interactions between dUba2, lwr and dPias. Based on these data we propose that sumoylation has a role on Dmp53 function.

Another Dmp53-interacting partner is the *Daxx-like protein (DLP),* the homologue of the human Daxx protein, which plays a role in Fas-mediated apoptosis and transcriptional repression. Daxx has been shown to bind p53, but the effects of this interaction are controversial. To gather more information about *DLP,* we generated loss of function mutants by P element remobilization. We found that *DLP* is not essential as homozygous mutants are viable and fertile, although their longevity is reduced. Moreover, we observed the effect of Dmp53 overexpression in flies on DLP mutant and wild-type background and found that the DLP mutant animals die earlier indicating that DLP acts as a repressor of p53 functions.

Supervisor: Imre M. Boros
E-mail: pardi@brc.hu

Heavy metal-induced genes in Synechocystis sp. PCC 6803. Applications for biosensor development

Loredana Peca

Institute of Plant Biology, Biological Research Center, Hungarian Academy of Sciences, Szeged, Hungary

The aim of the study is to characterize the transcriptional response of *Synechocystis* sp. PCC 6803 to metalloid and heavy metal stress, to find potential candidates for biosensing applications and to develop recombinant whole-cell sensors by coupling the metal-specific genes with bacterial luciferase genes.

A series of small-scale studies were conducted with a set of selected ORFs: slrl457, slr0944, slll957, slrl950, slll920, ssr2857, slr0798, slr0797, slr0793. The cells were incubated with biologically relevant concentrations of Co II (3 pM), Zn II (6 pM), Ni II (15 pM), Cd II (1.5 pM), Cr III (50 pM), Cr VI (50 pM), As III (ImM). As V (ImM) and Cu II (lpM) and the gene expression was assessed by quantitative RT-PCR.

ORF slr0798 *(ziaA)* that encodes a putative Zn II efflux P-type ATPase (Thelwell et al. 1998) showed a markedly increase in mRNA level after 15 min incubation with Cd II (49x) and arsenic ions (50x-As III and 18x-AsV), besides the known induction by Zn II (30x). ORF slr0797 *(coaT)* encodes a putative Co II translocating P-type ATPase (Rutherford et al.1999) and was strongly induced in our experiments by Co Hand Zn II(=° 15x), moderately induced by As III (5x), and weekly induced by Cd II (3x). Expression of ORF slr0793 *(nrsB)* that is involved in Ni II and Co II efflux (Lopez-Maury et al. 2002) was highly induced by Ni II (350x) and to a low extent by Co II and ZnII(=8x).

Synechocystis sp PCC 6803 contains an arsenic and antimony resistance operon *arsBHC,* under the control of a transcription repressor encoded by a distinctly located *arsR* (Lopez-Maury et al. 2003). We showed that ORF *slr0944 (arsB)* was highly induced after 15 min incubation with arsenic ions (1800x-As III and 700x-As V) and ORF slll957 *(arsR)* displayed no significant modification of its transcript level. ORFs *atxl* (ssr2857), *ctaA* (slrl950) and*pacS* (slll920) encode copper-trafficking determinants. The Atxl copper metallochaperone interacts with P(l)-type copper ATPases CtaA and PacS to supply copper proteins within intracellular compartments (Tottey et al. 2002). We could detect no significant transcript level modifications for these ORFs after exposure to the previously mentioned metal concentrations.

The predicted protein product of ORF slrl457 *(chrA)* belongs to the CHR family of prokaryotic transporters probably encoding chromate/sulfate antiporters. In our experiments *chrA* transcript level was not substantially modified by 7 pM, 20 pM and 50 pM Cr III or Cr VI added to BG-11 medium.

Two recombinant strains were constructed in our laboratory: strain *nrsB::luxAB* that showed specificity for Ni II and the bioluminescent signal was proportional to the metal concentration within the range 0.5-17 pM and strain *coaT::luxAB* that responded to both Co II and Zn II, proportional to the metal concentration between 0.2 and 4 pM, with a higher response for Co II. The recombinant strains activity is to be tested on environmental samples containing known concentration of Ni II, Zn II or Co II.

ORF slr0944 *(arsB)* is specifically induced by arsenic ions and represent another good candidate for biosensing applications.

López-Maury L, García-Domínguez M, Florencio FJ, Reyes JC (2002) A two-component signal transduction system involved in nickel sensing in the cyanobacterium Synechocystis sp. PCC 6803. Mol Microbiol 43(l):247-256.

Lopez-Maury L, Florencio FJ, Reyes JC (2003) Arsenic sensing and resistance system in the cyanobacterium Synechocystis sp strain PCC 6803. J Bacteriol. 185: 5363-5371.

Rutherford JC, Cavet JS, Robinson NJ (1999) Cobalt-dependent transcriptional switching by a dual-effector MerR-like protein regulates a cobalt-exporting variant CPx-type ATPase. J Biol Chem 274:25827-25832.

Thelwell C, Robinson NJ, Turner-Cavet JS (1998) An SmtB-like repressor from Synechocystis PCC 6803 regulates a zinc exporter. Proc Natl Acad Sci USA 95: 10728-10733.

Tottey S, Rondet SA, Borrelly GP, Robinson PJ, Rich PR, Robinson NJ (2002) A copper metallochaperone for photosynthesis and respiration reveals metal-specific targets, interaction with an importer, and alternative sites for copper acquisition. J Biol Chem 277(7):5490-5497.

Supervisor: Imre Vass
E-mail: loredana@brc.hu

New approaches in microbial degradation of mycotoxins

Zsanett Adrienn Péteri

Department of Microbiology, University of Szeged, Szeged, Hungary

Mycotoxin contamination of agricultural products is a serious health hazard throughout the world. One of the most important mycotoxins is ochratoxin A (OTA), which is produced by several *Aspergillus* and some *Penicillium* species. The occurrence of OTA in several commodities (feeds, foods and beverages) is considered as a serious health hazard in view of its nephrotoxic, teratogenic, hepatotoxic and carcinogenic properties.

Several strategies are available for the detoxification of mycotoxins. These can be classified as physical, chemical, physicochemical and (micro)biological approaches. Microbes or their enzymes could be applied for mycotoxin detoxification; such biological approaches are now being widely studied. An adsorption mechanism has also been suggested for OTA removal by lactic acid bacteria, yeasts (Bejaoui et al. 2004) and conidia of black aspergilli (Bejaoui et al. 2005). We examined *Phaffia rhodozyma* and *Xanthophyllomyces dendrorhous* isolates for their ability to degrade and/or adsorb ochratoxin A in a liquid medium (Péteri et al. 2006). *Phaffia rhodozyma* is a red-pigmented fermentative yeast. Besides producing astaxanthin, *P. rhodozyma* is also able both to detoxify and adsorb OTA at temperatures well above the temperature optimum for growth of *Phaffia* cells. We also examined several filamentous fungi representing the genera *Aspergillus, Rhizopus* and *Mucor* for their ability to degrade ochratoxin A i n a liquid medium (Varga et al. 2005).

The kinetics of OTA degradation of *P. rhodozyma* CBS 5905 has been examined at two cell concentrations at 20°C in a liquid medium. The *Phaffia* isolates could degrade more than 90% of OTA in about 7 days at 20°C. Previously, an A. *niger* isolate CBS 120.49 and *Actinomucor elegáns* NRRL 3104, *Rhizopus stolonifer* TJM 8A8 were found to be able to degrade more than 90% of OTA after *4* and 10 days incubation. Interestingly, a significant amount of OTA was found to be bound by the cells after two days, indicating that OTA is also adsorbed by the cells.

When the effect of temperature was examined, the temperature optimum of this enzyme was found to be above 30°C, which is much higher than the temperature optimum for growth of *P. rhodozyma* cells, which is around 20°C, and the cells are unable to grow at higher temperatures. When the temperature range of the OTA degrading enzyme was further examined, it was found that the enzyme remains active at up to 60°C. Above this temperature, OTA adsorption only could took place.

We hypothesized that a carboxypeptidase enzyme could be responsible for OTA degradation as observed previously in other fungi. To prove this hypothesis, the effect of various carboxypeptidase inhibitors was tested on OTA degradation activities *oiP. rhodozyma* cells. Two of these inhibitors, the chelating agents EDTA and 1,10-phenanthroline inhibited significantly OTA degrading activities of *theP. rhodozyma* cells, indicating that the enzyme responsible for OTA degradation is a metalloprotease.

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Further studies are in progress to identify the enzymes and genes responsible for ochratoxin detoxification (for example carboxypeptidase A), and to transfer these genes to yeast and other fungal isolates.

Bejaoui H, Mathieu F, Taillandier P, Lebrihi A (2004) Ochratoxin A removal in synthetic and natural grape juices by selected oenological Saccharomyces strains. J Appl Microbiol 97:1038-1044.
Bejaoui H, Mathieu F, Taillandier P, Lebrihi A (2005) Conidia of black aspergilli as new biological adsorbents for ochratoxin A in grape juices and musts. J Agrie Food Chem 53:8224-8229.
Péteri Zs, Téren J, Vágvölgyi Cs, Varga J (2006) Ochratoxin adsorption caused by astaxanthin-producing yeasts. Food Microbiol (in press).
Varga J, Péteri Zs, Tábori K, Téren J, Vágvölgyi Cs (2005) Degradation of ochratoxin A and other mycotoxins by Rhizopus isolates. Int J Food Microbiol 99:321-328.

Supervisors: Csaba Vágvölgyi, János Varga
E-mail: zspeteri@gmail.com

The interaction of lysyl oxidase with the hormone placental lactogen and their effect on mammary epithelial cell proliferation and migration

Noémi Polgár

Cardiovascular Research Center, JABSOM, University of Hawaii, Honolulu, Hawaii, USA, Department of Genetics, University of Szeged, Szeged, Hungary

Lysyl oxidase (LOX), a copper-dependent amine oxidase, contributes to the assembly and maintenance of the extracellular matrix (ECM) by initiating the formation of covalent cross-links in collagen and elastin (Smith-Mungo et al. 1998). Besides its matrix-stabilizing function, LOX has recently been shown to play a role in cell motility, transcriptional regulation, embryonic development and pathological conditions such as breast cancer (Csiszár et al. 2001). While the ECM cross-linking activity of LOX is well studied, the mechanisms of the novel functions are not known. To identify protein interactions that determine and/or regulate these LOX activities, we performed a yeast two-hybrid screen using a human placental cDNA library and both full-length and mature LOX as baits, and identified placental lactogen (PL) as a possible interacting partner. PL, a member of the growth hormone (GH)-prolactin (PRL) hormone family, stimulates mammary gland development, lactogenesis and the growth and metabolism of the foetus (Walker et al. 1991). PL is only expressed in the placental syntitiotrophoblasts, but its expression was shown in 77% of invasive ductal carcinomas, and the amplification of the PL genes has been reported in 22% of the cases (Latham et al. 2001). While PL was reported to promote epithelial cell proliferation in breast carcinomas, its role in breast tumours is not fully understood.

Our direct interaction studies, Far-Western analysis and solid phase binding assays supported LOX binding to PL and suggested binding to GH as well. In addition, *in vitro* amine oxidase activity assays showed that PL is neither a substrate nor an inhibitor of LOX. Since increased expression and enzyme activity of LOX have been reported in highly invasive and metastatic breast cancer cell lines as well as in metastatic breast tumours, and PL has been shown to be expressed in breast carcinomas, we tested their expression in tissue sections of mammary carcinomas and breast cancer cell lines. Using fluorescence-labelled immunostaining on a tissue macroarray, we detected LOX and PL expression in and around tumour cells. Subsequently, we tested protein expression of breast cancer cell lines, and found elevated PL expression in highly invasive MDA-MB231 and Hs578T cell lines, where LOX expression was also increased. Furthermore, we showed PL expression in poorly invasive MCF-7 and T47D cells at elevated and low levels, respectively. Since the highly invasive and metastatic breast cancer cell lines express both PL and LOX, we decided to study their individual and combined effects on cell behaviour by overexpressing and coexpressing these proteins in immortalized normal breast epithelial cells. LOX plays a role in promoting cell migration, while PL was shown to induce cell proliferation, thus we tested these processes. Stably transduced MCF-10A normal mammary epithelial cells coexpressing PL and LOX had significantly increased proliferation rates compared to the parental and the PL-expressing cells, while LOX alone had no effect on proliferation. Therefore, the coexpression of LOX with PL appears to enhance the proliferation-inducing effect of PL. Coexpressing cells in addition showed a significantly higher migratory rate compared to cells expressing either or none of these proteins. Our results demonstrated that LOX, in addition to promoting tumour cell invasion through a H_20_2-induced FAK/Src activation (Kirschmann et al. 2002), may further induce tumour cell migration in interaction with PL by activating independent signalling.

Csiszár K (2001) Lysyl oxidases: a novel multifunctional amine oxidase family. Prog Nucleic Acids Res Mol Biol 70:1-32.
Kirschmann DA, Seftor EA, Fong SF, Nieva DR, Sullivan CM, Edwards EM, Sommer P, Csiszár K, Hendrix MJ (2002) A molecular role for lysyl oxidase in breast cancer invasion. Cancer Res 62:4478-4483.
Latham C, Zhang A, Nalbanti A, Maner S, Zickert P, Blegen H, Zetterberg A (2001) Frequent co-amplification of two different regions on 17q in aneuploid breast carcinomas. Cancer Genet Cytogenet 127:16-23.
Smith-Mungo LI, Kagan HM (1998) Lysyl oxidase: properties, regulation and multiple functions in biology. Matrix Biol 16:387-398.
Walker WH, Fitzpatrick SL, Barrera-Saldana HA, Resendez-Perez D, Saunders GF (1991) The human placental lactogen genes: structure, function, evolution and transcriptional regulation. EndocrRev 12:316-328.

Supervisors: Katalin Csiszár, Mátyás Mink
E-mail: polgar.noemi@gmail.com

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Genetic and molecular analysis of the *Drosophila* CalpA and CalpB

Ferenc Sandor Pop

Institute of Genetics, Biological Research Center, Hungarian Academy of Sciences, Szeged, Hungary

Calpains are Ca2+ -activated cytoplasmatic SH-proteases, that through a limited proteolysis of their substrate proteins, regulate apoptosis, the cell cycle and many cellular pathways. From the four calpain genes found in the Drosophila genome, only two (CalpA and CalpB) code for canonical and functional calpain proteins (Friedrich et al. 2004). Our aim was to study the functions of these two genes, by creatind mutants with the remobilization of the P-elements found in their vicinity.

Based on our and other groups (Grabbe et. al 2004) results, we concluded that the CalpA gene is not essential and its alteration does not lead to a lethal fenotype. Based on this, we remobilized the EY10816 P-element (Voelker RA et al. 1984), and screened the resulting CalpA mutant candidates for homozigous viable mutants. Using Anti-CalpA primary antibody we examined the expression of the CalpA protein during the different developmental stages of the Drosophila ontogenesis. In the embryos the CalpA protein is mainly expressed in the CNS, while in the later larval stages the expression of the protein is reduced to only a few neurons. In the Drosophila ovaries, the CalpA protein is located at the membranes and in the nuclei of the germ- and follicular cells. The protein is also present in the testes, where it is located only at the membranes of the spermatogonial cells, while in the spermatocytes and spermatids it can be found both at the membranes and nuclei of the cells. In the ring gland and larval fat body, the CalpA protein exibits the same nuclear and membranar expression pattern.

With the help of EP972 P-element we created homozigous lethal CalpB mutants and analysed the molecular breakpoints of five candidates, but regretably all of them were double mutants, which means that the adjacent Taf2 gene was also affected by the deletions. Our group performed another remobilization using a different P-element, the EY08042. This time we created five homozigous viable CalpB mutants, in which the deletions did not affect the adjacent Taf2 gene.

These findigs suggest that the Drosophila CalpA and CalpB do not play an essential role. We were curious what would happen, if we alterd their function in the same time, and we found, that combining the different CalpA and CalpB homozigous viable mutants resulted in lethality and sterility, which indicates the redundancy of these genes.

Friedrich P, Tompa P, Farkas A (2004) The calpain system in Drosophila melanogaster: coming ofage. BioEssays 26:1-9.

Grabbe C, Zervas CG, Hunter T, Brown NH, Palmer RH (2004) Focal adhesion kinase is not required for integrin function or viability in Drosophila. Development 131(23):5795-5805.

Voelker RA, Greenleaf AL, Gyurkovics H, Wisely GB, Huang S, Searles LL (1984) Frequent imprecise excision among reversions of a P-element-caused lethal mutation inDrosophila. Genetics 107:279-294.

Supervisor: Géza Ádám
E-mail: popf@brc.hu

Role of the estrogen caused up-regulation of heme-oxygenase enzyme in the protective mechanism of cardiovascular system

Anikó Pósa

Department of Comparative Physiology, University of Szeged, Szeged, Hungary

Gender-based differences in the incidence of hypertensive and coronary artery disease, the development of atherosclerosis, and myocardial remodelling after infarction are attributable to the direct effect of estrogen on the myocardium, vascular smooth muscle and endothelium. In the heart and vasculature, these mediate rapid vasodilatation (White et al. 1997), reduces both myocardial infarct size and the occurrence of ischemia- and reperfusion-induced ventricular arrhythmias in hearts (Node et al. 1997).

Since the effects of hormone replacement therapy on the risk of cardiovascular events are controversial, and treatment with estrogen in high dose can cause breast and endometrial carcinoma, selective estrogen receptor modulators (SERMs) have come to the focus of attention. Raloxifene (RAL), a second generation selective estrogen receptor modulator (SERM), is of use for the treatment of osteoporosis. RAL acts as an estrogen agonist in cardiovascular system but as an estrogen antagonist in breast and endometrium. RAL is currently being assessed for menopausal women at risk of ischemic heart disease (Raloxifene Use for the Heart Trial). According to several reports, nitric oxide (NO) plays an important role in mediating the beneficial effects of estrogen and raloxifene in the vascular system. NO is a well established effector molecule of estrogen and SERM mediated vasoprotection (Pávó et al. 2000)

Heme-oxygenase (HO) is the rate-limiting enzyme for heme degradation in mammals. To date, three isoforms of HO have been characterized: HOI is widely expressed and is inducible by a range of stimuli that produce oxidative stress. H02 is non inducible form, it occurs in neuronal populations and vascular endothelial cells, while H03 is newly recognized form. We attempted to clarify the effects of estrogen and raloxifene modulated HOI and H02 enzymes in rat cardiac.

Hypothesis: Treatment with 17-Ebestradiol or SERM raloxifene increases the activity of heme- oxigenase (HO) and the expression of H02 in the ventricle and decreases the arginin vasopressin (AVP) induced increase of blood pressure and heart perfusion in experimental menopause.

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Methods: In our experiments intact females in oestrus cycle, ovariectomized (OVX), RAL or 17-p oestradiol treated OVX female Wistar rats were used. HO enzyme system was inhibited by tin-protoporphyrin (SnPP; 30 mg/kg, s.c.). In all groups we examined 1. / the activity of HO and expression of HOI and H02 in the ventricle, 2./ the ST depression (standard lead II surface ECG) *in vivo,* 3./ the increase in blood pressure *in vivo* and heart perfusion *ex vivo* induced by AVP.

Results: Ovariectomy 1. / decreased HO activity (from 2.2 nmol bilirubin/h/mg protein to 0,9 nmol bilirubin/h/mg protein); HOI and H02 expression (46 ± 4% and 47 ±3 % respectively) in the ventricle, 2. / increased the tendency of the heart ischemia (ST segment change: from -0,02 mV- -0,13 mV), increased the response of blood pressure (15-25%) and heart perfusion (10-30%) to AVP, as compared to intact females. 17-p oestradiol and RAL replacement restored the difference induced by OVX to the level observed in the ovary-intact females. SnPP treatment intensified the response of blood pressure (intact female 25-30%; OVX 10-12%; 17- p oestradiol 20-25%; RAL 23-28%) and heartperfusion (intact female 10%; OVX 5%; 17- p oestradiol 12%) to AVP.

Discussion: Presumably raloxifene can also diminish the increase in blood pressure induced by menopause by the augmentation of HO synthesis. In the system applied, raloxifene had estrogen agonistic effect.

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Node K, Kitakaze M, Kosaka H, Minamino T, Funaya H, Hori M (1997) Amelioration of ischemia- and reperfusion-induced myocardial injury by 17beta-estradiol: role of nitric oxide and calcium-activated potassium channels. Circulation 96(6):1953-1963.

Pavo I, László F, Morschl E, Nemesik J, Berkó A, Cox DA, László FA (2000). Raloxifene, an estrogen receptor modulator, prevents decreased constitutive nitric oxide and vasoconstriction in ovariectomized rats. Eur J P 410:101-104.

White CR, Shelton J, Chen SJ, Darley-Usmar V, Allen L, Nabors C, Sanders PW, Chen YF, Oparil S (1997). Estrogen restores endothelial cell function in an experimental model of vascular injury. Circulation 96(5):1624-1630.

Supervisors: Ferenc László, Csaba Varga
E-mail: paniko@bio.u-szeged.hu

Positional cloning of the rough coat mice; molecular analysis of a novel predicted adhesion molecule

Peter Racz

Department of Genetics, University of Szeged, Szeged, Hungary, Cardiovascular Research Center, University of Hawaii, Honolulu, USA

The rough coat *(rc)* phenotype arose spontaneously as a recessive trait in C57BL/6J mice. Homozygous *rc* mice were characterized with an unkempt-looking hair coat by weaning age, cyclic and progressive hair loss and sebaceous gland hypertrophy. Previously, the *rc* locus was mapped to a 4-centimorgan region (at 32.0 centimorgan) on chromosome 9, close to the *Mpi-1* gene at 57 Mb (Ensembl Mouse Genome Database v38) but the gene mutation remained unidentified.

To understand the genetic basis of the rc phenotype, we carried out positional cloning in backcross mice. Our research group outcrossed B6J-*rc/rc* mice with both CAST/Ei mice and Balb/cJ mice to obtain Fj hybrids (*+/rc)* on two mixed strain backgrounds to compensate for a potential low rate of recombination within the *rc* region. Female Fj hybrids were backcrossed with male B6J-*rc/rc* mice to obtain F_2 hybrids. We analyzed linkage between the rc locus and published microsatellite polymorphisms (Mouse Genome Informatics "Strains and Polymorphisms" database (www.informatics.jax.org) or the Ensembl Mouse Genome Database (www.ensembl.org/Mus_musculus)) between the parental strains, within the 4-centimorgan region on chromosome nine using 700 B6J-Balb/cJ F_2 hybrids. We reduced the rc interval to a 1.54 Mb region, between D9Mit228 (44.13Mb) and D9Mitl92 (45.67Mb). Within this interval, we identified 18 novel microsatellite polymorphisms between B6J and Balb/cJ strains. Using 361 B6J-CAST/Ei F2 hybrids, my collaborators reduced the rc interval to 246 kb, between 44.83Mb and 45.0796 Mb. Within this 246-kb region, there are 11 candidate genes (Ensembl Mouse Genome Database v38). After analyzing all the coding exons and flanking splice sites by sequence analysis of PCR products using wild type B6J and B6J-*rc/rc* genomic DNA as templates, a G^A transition was identified in the coding sequence of a novel gene ENSMUSG00000070305 (44.989-45.009Mb).

The highest homology of this gene are to Myelin Protein Zero (MPZ) and Myelin Protein Zero-like 2 (MPZL2, also called Epithelial V-like Antigen or EVA1). We therefore named this gene *Mpzl3* (Myelin Protein Zero-like 3).

The *Mpzl3* gene contains at least six exons, and gives rise to at least two transcripts through alternative splicing. A two-exon transcript encodes a 91 amino acid polypeptide, and a six-exon transcript encodes a 237 amino acid polypeptide.

RT-PCR based expression pattern confirmed that both *Mpzl3* transcripts are expressed in a variety of organs with high levels in the brain, heart, liver and the skin.

Bioinformatical analysis of the predicted MPZL3 protein revealed a cell adhesion molecule with signal peptide, two transmembrane domains and a highly conserved immunoglobulin domain, in which the point mutation was found to affect a conserved residue.

To begin to identify tissue expression and localization of this protein, we carried out bioinformatical analyses and indirect immunofluorescence assay. The results confirmed our hypothesis that this protein might be a membrane protein expressed in multiple tissues.

In our future studies we are going to focus on the human homologue of the Mpzl3 gene in order to identify potential mutations and the corresponding human phenotype(s) linked to these gene mutations. Our data provide insight into the role of novel *Mpzl3* gene and help to better understand the molecular mechanism which cause the *rc* phenotype.

Cao T, Racz P, Szauter KM, Groma G, Nakamatsu GY, Fogelgren B, Pankotai E, He QP, Csiszár K (2007) Mutation in , a Novel Gene Encoding a Predicted *Mpzl3*
Adhesion Protein, in the rough coat *(rc)* Mice with Severe Skin and Hair Abnormalities. J Invest Dermatol 2007 Feb 1; [Epub ahead of print] PMID: 17273165.
Hayashi K, Cao T, Passmore H, Jourdan-Le Saux C, Fogelgren B, Khan S, Homstra I, Kim Y, Hayashi M, Csiszár K (2004) Progressive hair loss and myocardial degeneration in roughcoat mice: reduced lysyl oxidase-like (LOXL) in the skin and heart. J InvestDermatol 123:864-871.

Supervisors: Tongyu Cao, Katalin Csiszár, Mátyás Mink
E-mail: petiracz@yahoo.com

Investigation of neuroprotection in different models

Gabriella Rákos

Department of Comparative Physiology, University of Szeged, Szeged, Hungary

Trauma in general, and head injury in particular, is the most frequent cause of mortality and morbidity. The outcome of a severe head injury depends on the severity of the primary lesion and the manifestations of secondary brain damage. Brain edema and the secondary growth of traumatic brain tissue, necrosis, are important manifestations of secondary brain damage.

The cold injury model is one of the established models for study of disruption of the blood-brain barrier (BBB) and vasogenic brain edema development. A cold lesion was induced by applying a precooled (-78°C) copper cylinder for 30 s to the intact skull of rats. This cold lesion induces a dysfunction not only of the BBB, but also of the cellular membranes. This may induce a secondary neuronal loss in the perilesional rim, the main target of neuroprotective interventions. The non-intact cells can be detected by markers of apoptosis only hours or even days after injury.

The dye Evans blue (EB) is known to bind to serum albumin after intravenous injection and has been used as a tracer of serum albumin. The early membrane dysfunction allows extravasated serum proteins and their tracers, such as EB, to enter the cells, permitting their early visualization.

The aim of our work was to demonstrate injured cells that take up EB in the perilesional rim. In cold-lesioned animals, the extravasated EB content in the injured hemisphere was highest 0.5 h after EB administration (Murakami et al. 1999). Accordingly, there is hope that we can obtain information on the cortical extent of the area of non-intact cells much earlier than with other immunohistochemical methods. In our study, EB-positive cells were detected in the perilesional rim. These cells emitted bright-red autofluorescence and could easily be counted (Rákos et al. 2007). This method proved a useful tool in pilot experiments performed to test the presumed neuroprotective effects of candidate agents in the cold lesion model (Juhasz-Vedres et al. 2006)

We used another model to produce focal cerebral ischemia. Ischemic stroke is also a leading cause of death and disability. The photothrombotic model for stroke was originally described as a focal cortical infarction resulting from occlusive thrombosis. In this model, through the use of transcranial illumination with a cold light source in combination with the intravenous injection of rose bengal, a potent photosensitive dye, it was possible to produce the thrombosis of small blood vessels. It is important to understand the cellular and molecular responses to cerebral ischemia in order to provide adequate therapeutic strategies for such injury. It is well known that elevated glutamate levels after cerebral ischemia play a key role in the development of neuronal damage.

We used Fluoro Jade, an anionic fluorochrome, to visualize the ischemic neuronal damage. The histochemical application of Fluoro Jade results in a simple and sensitive method for staining degenerating neurons.

In our study, we tested the prediction that oxaloacetate-mediated blood glutamate scavenging causes neuroprotection in the photothrombotic lesion model. The volume of the hemispheric lesion and the number of Fluoro Jade-positive cells were determined. The results demonstrated that the extent of the lesion and the number of Fluoro Jade- labeled cells were significantly smaller in the oxaloacetate-treated group. It is concluded that even a single posttraumatic administration of oxaloacetate may be of substantial therapeutic benefit in the treatment of focal brain injury.

Juhasz-Vedres G, Rózsa E, Rákos G, Dobszay M, Kis Zs, Wölfling J, Toldi J, Párducz A, Farkas T (2006) Déhydroépiandrostérone sulfate is neuroprotective when administered either before or after injury in a focal cortical cold lesion model. Endocrinology 147(2):683-686.
Murakami K, Kondo T, Yang G, Chen SF, Morita-Fujimura Y, Chan PH (1999) Cold injury in mice: a model to study mechanism of brain edema and neuronal apoptosis. Prog Neurobiol 57:289-299.
Rákos G, Kis Zs, Lür Gy, Farkas T, Hortobágyi T, Vécsei L, Toldi J (2007) Evans blue autofluorescence permits the rapid visualization of non intact cells in the perilesional rim (in press).

Supervisor: Zsolt Kis
E-mail: rakosgabi@gmail.com

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Growing or escaping: transcriptome analysis during long-term drought stress adaptation in two wheat *(Triticum aestivum L.)* genotypes

Maria Secenji

Institute of Plant Biology, Biological Research Center, Hungarian Academy of Sciences, Szeged, Hungary

Among environmental stresses, drought is a major abiotic factor that limits agricultural crop production. Nevertheless, water deficit usually coincides with other abiotic stresses, *i.e.* high temperature, high light intensity, salt stress, adversely effecting plant growth.

However, plants can survive these adverse conditions using different strategies. Plant resistance to water deficit may arise from escape, avoidance, or tolerance strategies (Levitt 1972). "Escapers" are able to complete their life-cycle before the onset of severe stress; drought avoidance relies on maintaining high tissue water potential by minimizing water loss and maximizing water uptake (Chaves et al. 2003) while drought tolerance is achieved by co-ordination of physiological and biochemical alterations at the cellular and molecular levels including specific gene expression and accumulation of specific proteins under drought stress. Plants usually apply a combination of these strategies under dry conditions.

To examine long-term drought stress adaptation of two wheat cultivare, both displaying tolerance to water-deficit stress but following distinct strategies, a greenhouse experiment was established including treated and non-treated wheat plants growing in perlite as a soil substitute. Treated samples were irrigated with one sixth the amount of irrigation solution causing a mild water-deficit stress to the plants. The presence and extent of the stress was confirmed by following the expression levels of L- '-pyrroline-S-carboxylate synthetase (P5CS), the key enzyme of proline biosynthesis, during the four-week-long treatment. Proline accumulation, the consequence of induced expression of P5CS, is a well-described phenomenon in plants subjected to drought stress (Yoshiba et al. 1997).

Furthermore, water deficit alters growth of shoots and roots, resulting in an increased root/shoot ratio. In this study, the adaptive genotype showed a much higher root/shoot ratio under water stress, due to favored root growth to shoot growth, compared to the "escaper" one.

To follow the transcriptional changes under water deficit, cDNA macroarray was hybridized with root samples from both genotypes. As a result, 8.0% of the genes were up-regulated and 8.5% were down-regulated in the adaptive cultivar. In the "escaper" genotype, these ratios were 5.1% and 4.8%, respectively.

Up- and down-regulated genes were clustered into six groups each, based on their temporal expression profiles. The clusters' functional classification was done based on the predicted function of the encoded proteins, according to a modified version of the categorization described by Yang et al. (2004).

After analyzing the classes of up-regulated genes, three of these groups showed significant differences between the two genotypes, referring to the possible genetic background of their strategies. In the "escaper" genotype, a considerable proportion of the up-regulated genes, encoding proteins endowed with predicted stress- and defense-related functions, was presented. However, in the adaptive genotype, the ratio of genes, encoding proteins involved in signal transduction and cell wall biogenesis, was higher than in the "escaper" one.

These results suggest a hypothetical elucidation of the molecular genetic background of the different tolerance strategies of the two examined wheat genotypes.

YoshibaY, Kiyosue T, Nakashima K, Yamaguchi-Shinozaki K, Shinozaki K (1997) Regulation of levels of proline as an osmolyte in plants under water stress. Plant Cell Physiol 38:1095-1102.

Levitt J (1972) Responses of plant to environment stress. Academic Press. N.Y.

Chaves MM, Maroco JP, Pereira JS (2003) Understanding plant responses to drought - from genes to the whole plant. Funct Plant Biol 30:239-264.

Yang L, Zheng B, Mao C, Qi X, Liu F, Wu P (2004) Analysis of transcripts that are differentially expressed in three sectors of the rice root system under water deficit. Mol Gen Genomics 272:433-442.

Supervisor: János Györgyey
E-mail: szecsma@brc.hu

Differential polarization laser scanning microscopy - development and biological applications

Gábor Steinbach

Institute of Plant Biology, Biological Research Center, Hungarian Academy of Sciences, Szeged, Hungary

Confocal microscopes are quite often used for 3D imaging of fluorescent samples. But the intensity measurements will give only partial information about the interaction between the material and the light: there are 16 parameter in the Mueller matrix describing the interaction, but the absorbance is only one of them.

We have constructed a differential polarization (DP) attachment for imaging linear dichroism (LD) fluorescence detected linear dichroism (FDLD) and other DP values with our Zeiss 410 laser scanning microscope. The attachment uses high frequency modulation and subsequent demodulation, via lock-in amplifier, of the detected intensity values, and displays pixel-by-pixel the measured DP quantity. LD

carries information on the orientation of the dye molecules, more exactly on the anisotropic distribution of the absorbance transition dipole vectors of the chromophores, in the sample. FDLD carries the same information for fluorescent dyes: the fluorescence intensities excited with orthogonally polarized light are being proportional to the number of the absorbed quanta. Due to the confocal fluorescent measurement, 3D anisotropic distribution of the transition dipoles can also be reconstructed.

Demonstration was performed on two different samples - that LD and FDLD images, specially after the performed 3D reconstruction are able to carry detailed information about the molecular organization: on sections of Convolaria majalis root tissue stained with Acridine Orange provide quantitative information on the anisotropy of cell wall. LD images, in non-confocal mode, appear to indicate that the cell walls exhibit weak anisotropy. However, confocal FDLD images, due to the thin optical sections, reveal that these highly organized fiber-laminate extracellular structures exhibit very strong local anisotropy values. A simple mathematical model shows that the magnitude of FDLD depends on the intercalation angle between the dye molecule and the fiber and the angular distribution of the fibers with respect to their preferential orientation, *i.e.,* with respect to the cell walls

Amyloid fibrils, insoluble protein aggregates, are associated with a large variety of diseases. When stained with an intercalating dye, such as Congo Red, the fibrils exhibit strong linear birefringence due to the highly ordered molecular architecture of the self-assembled bundles. By using the differential polarization laser-scanning microscope, we investigated the spatial distribution of optical anisotropy properties of isolated human amyloid fibrils stained with Congo Red and Rivanol, and investigated the fine structure of the fibrils.

Juang CB, Finzi L, Bustamante CJ (1988) Design and Application of a Computer-Controlled Confocal Scanning Differential Polarisation Microscope. Rev Sci Instrum 59:2399-2408.

Mickols WC, Bustamante C, Maestre MF, Tinoco I, Embury SH (1985) Differential Polarization Microscopy: A New Imaging Technique. Bio-Technology 3:711-714.

Steinbach G, Pomozi I, Javorfi T, Menczel L, Zsiros O, Gorjanacz M, Gombos I, Kiss I, Matko J, Makowitzky J, Garab G (2005a) Highly organized molecular macroassemblies imaged by differential polarization laser scanning microscopy. FEBS J 272:446-447.

Steinbach G, Besson F, Pomozi I, Garab G (2005b) Differential polarization laser scanning microscopy: biological applications. Proceedings of the SPIE 5969:566-575.

Steinbach G, Pomozi I, Garab G, Makovitzky J (2006) Periodic twisted structure of amyloid fibrils revealed by differential polarization laser scanning microscopy. FEBS J 273:65-65.

Supervisor: Győző Garab
E-mail: stein@brc.hu

Diurnal regulation of brassinosteroid biosynthesis and perception

Anna-Mária Szatmári

Institute of Plant Biology, Biological Research Center Hungarian Academy of Sciences, Szeged, Hungary

Brassinosteroids (BRs) are recently recognized polyhydroxylated steroid hormones that are important regulators of plant growth and development. Their physiological functions were determined by using brassinosteroid-deficient mutants. In addition to the role of these phytohormones in promoting growth, fertility, and stress resistance, they also regulate photomorphogenesis (Li et al. 1996; Szekeres et al. 1996). BRs are known to act at, or near, the sites of their synthesis, therefore the regulation of BR biosynthesis can directly control local physiological effects.

BRs are synthesized from phytosterols through multiple, mostly oxidative steps leading to brassinolide (BL), the biologically most active BR. The oxidative reactions are catalyzed by closely related cytochrome P450 monooxygenases of the CYP85 or CYP90 families (Fujioka and Yokota 2003). Recent studies have indicated that in *Arabidopsis* the expression of all BR-biosynthetic P450 genes is under both developmental and organ-specific regulation that takes place primarily at the level of transcription. In addition, all these genes are under negative feedback regulation by active BRs (Mathur et al. 1998; Bancos et al. 2002). Therefore, it seemed likely that the activities of genes encoding rate-limiting enzymes, such as *CPD (CONSTITUTIVE PHOTOMORPHOGENESIS AND DWARFISM)* and *CYP85A2* can directly influence the efficiency of biosynthesis and hormone level. In an attempt to monitor the expression of these key biosynthetic genes, we generated transgenic plants carrying promoter:luciferase fusions, and followed gene activities on the basis of luciferase-generated *in vivo* luminescence. We found that *CPD* activity displays a complex diurnal pattern, with maxima following the onset, and coinciding with the end of light periods. This expression profile is determined by a circadian oscillation and a superimposed positive light regulation. Very similar daily expression cycles were observed with *CYP85A2,* the gene encoding the enzyme that produces BL.

To characterize the nature of light signaling, we determined the photoreceptor dependence of light induction. The severely decreased expression level of *CPD* in phytochrome-deficient background and the red light-specific induction in wild type plants suggest that the light regulation of *CPD* is mediated primarily by phytochrome signaling.

The diurnal periodicity of *CPD* activity is maintained in the BR insensitive *bril (brassinosteroid insensitive 1)* mutant, indicating that the underlying regulation is independent of the changes in the endogenous BR level. But we also observed that BR regulation is an important modulator of the diurnal expression pattern, being responsible for the repression of *CPD* in the dark.

In order to find out whether diurnal fluctuations of *CPD* and *CYP85A2* expression is accompanied by changes in the levels of bioactive BRs, we analyzed the BR content of *Arabidopsis* seedlings during the day using gas chromatography-coupled mass spectrometry. We

found a major, transient increase of the BL content in the middle of the light period, in good coincidence with the light activation of the key BR biosynthetic genes. Our results, therefore, suggest that the level of bioactive BRs is dependent on the diurnal changes in *CPD* and *CYP85A2* expression.

Bancos S, Nomura T, Sato T, Molnár G, Bishop GJ, Koncz C, Yokota T, Nagy F, Szekeres M (2002) Regulation of transcript levels of the Arabidopsis cytochrome P450 genes involved in brassinosteroid biosynthesis. Plant Physiol 130:504-513.

Fujioka S, Yokota T (2003) Biosynthesis and metabolism of brassinosteroids. Annu Rev Plant Biol 54:137-164.

Li J, Nagpal P, Vitart V, McMorris TC, Chory J (1996) A role for brassinosteroids in light-dependent development of Arabidopsis. Science 272:398-401.

Mathur J, Molnár G, Fujioka S, Takatsuto S, Sakurai A, Yokota T, Adam G, Voigt B, Nagy F, Maas C, Schell J, Koncz C, Szekeres M (1998) Transcription of the Arabidopsis CPD gene, encoding a steroidogenic cytochrome P450, is negatively controlled by brassinosteroids. Plant J 14:593-602.

Szekeres M, Németh K, Koncz-Kálmán Z, Mathur J, Kauschmann A, Altmann T, Rédei G, Nagy F, Schell J, Koncz C (1996) Brassinosteroids rescue the deficiency of CYP90, a cytochrome P450, controlling cell elongation and de-etiolation in Arabidopsis. Cell 85:171-182.

Supervisor: Miklós Szekeres
E-mail: annama@brc.hu

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Acta Biologica Szegediensis, Editorial Office
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Monod J, Changeux J-P, Jacob F (1963) Allosteric proteins and cellular control systems. J Mol Biol 6:306-329.

Schwarz-Sommer Z, Huijser P, Nacken W, Saedler H, Sommer H (1990) Genetic control of flower development by homeotic genes in *Antirrhinum majus*. Science 250:931-936.

Article by DOI

Weiss E (2012) Examining activity patterns and biological confounding factors: Differences between fibrocartilaginous and fibrous musculoskeletal stress markers. Int J Osteoarchaeol DOI: 10.1002/oa.2290.

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Book chapter

Coons AH (1978) Fluorescent antibody methods. In Danielli JF, ed., General Cytochemical Methods. Academic Press, New York, 399-422.

Online document

Benny GL (2009) Zygomycetes. Available: http://www.zygomycetes.org. Accessed 11 November 2010.

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