Increased regeneration efficiency of ***Brassica napus*** L. cultivars Star, Westar and Cyclone from hypocotyle and cotyledonary explants

Israr Khan[1], Waqar Ali[2], Zahir Ali Takar[3], Arfa frooqi[4], Sikandar[5],[6]Waheed Akhtar

[1,2,3,4,5] Department of Biotechnology, University of Malakand
[6] School of Biological Sciences, University of the Punjab

Abstract

The comparative organogenesis of *Brassica napus* L cultivars Cyclone, Star and Westar was studied. The cotyledonary explants gave a higher response to all the combinations of 0.5 mg/L 2,4-D and BAP (0.5, 1.0,1.5 and 2.0 mg/L} used for optimizing the conditions for callus induction. The best mean weight and mean length of callus was obtained at 0.5 mg/L 2,4-D and 1.5mg/L BAP for Star cotyledonary explants. For the complete plant regeneration the new method of exposing the explants culture to Growth regulator free medium was performed. The method was applicable to both hypocotyl and cotyledonary explants. The Shoot Induction Frequency for hypocotyl (6-34%) in the three cultivars is higher than the cotyledonary explants (3-23 %.). The method is speedy and almost all of the shoots and some unshooted calli (78%) form roots on the same media without prior transfer to rooting medium. The Analysis of variance ($P<0.05$) showed that the data is significantly different and all the variation is due to the different groups/sources in the experiments. Thus it is recommended to use separate explants of each variety for gene transformation specially hypocotyl explants of westar.

Key words: Tissue culture, Regeneration, *Brassica napus*, Hypocotyle, Cotyledon

Introduction

The genus *Brassica* includes some of the very important crop species (Knutzon *et al.,* 1992) and is one of the most economically important genus in the *Brassica*ceae family (syn. Cruciferae). The Brassicaceae family comprises about 3000 species (Neeser *et al*., 1999). *Brassica* vegetables contain little fat, and are sources of vitamins, minerals and fiber. They also contain a large number of novel phytochemicals, some of which protect against carcinogenesis. (Willcox *et al*., 2003). *Brassica napus* ranks the third among the oil crops, following palm oil and Soya oil and the fifth among economically important crops, following rice, wheat, maize and cotton (Cardoza and Stewart, 2003). The seed contains up to 45% of an edible semi-drying oil. It is also used as a luminant or lubricant in soap making. (Greville, 2005). Canola is the Canadian oil association trademark which commonly refers to oil seed rape or any rapeseed, which is most often B. napus (Cardoza and Stewart, 2004) with less than 2% of erucic acid (C22:1) in the oil and less than 30 µmol of any one or all of the four major aliphatic glucosinolates named as 3-butenyle glucosinolate, 4-pentenyle glucosinolate, 2-hydroxy-3 butenyle glucosinolate and 2-hydroxy-4-pentenyle glucosinolates per gram of air dry oil free solid. Plant cell and tissue culture, also referred to as in vitro, axenic, or sterile culture is an important tool in both basic and applied studies and commercial application (Thorpe, 1990). The regulatory factors in the culture medium that regulate organogenesis include both naturally occurring and synthetic plant growth substances, as well as various environmental stimuli (Lakshmanan *et al*., 1997). With the increasing demand for canola oil, genetic engineering which reduces the time to develop a new variety has replaced conventional breeding and this technology mainly depends on tissue culture techniques.

Plant tissue culture technology has been successfully used for the commercial production of pathogen-free plants and to conserve the germplasm of rare and endangered species (Fay, 1992). Canola might also be especially useful as a vehicle to overproduce pharmaceutically active proteins and edible vaccines by applying the genetic engineering technology (Giddings *et al*., 2000).

MATERIALS AND METHODS

Plant materials

The hypocotyl and cotyledonary explants of three *Brassica napus* canola cultivars namely Cyclone, Star and Westar were used. The seeds of cultivar Cyclone were obtained from the National Agriculture Research Center (NARC) Islamabad while the seeds of cultivars Star and Westar were obtained from the Agriculture Research Station north Swat, N.W.F.P. The dark healthy seeds were selected and surface sterilized.

Seed surface sterilization and germination

The required number of seeds of the *Brassica napus cultivar* Cyclone were first washed by submerging them in water for one hour to remove the dust. The seeds were transferred to 70% ethanol for one minute followed by immersion in 0.01% (w/v) mercuric chloride for one minute. A few drops of Tween 20 were added as a surfactant and wetting agent to the mercuric chloride solution. Then the surface sterilized seeds were transferred to autoclaved distilled water and rinsed 2-3 times.The seeds were germinated in petri plates on 0.8% agar (w/v). Then incubated at 25±2°C in complete dark. After 2 days the seeds were transferred to a 16/8 h day/night photoperiodic regime under cool white fluorescent lights (1000-Lux) for 5 days.

Explants preparation

Cotyledons and hypocotyls segments from 7 days old seedlings were used as explants. These Hypocotyls 1-3 mm explants were carefully excised from the seedlings without including any of the meristematic axillary buds. About 25-30 seedlings per cultivar were used. The explants were readily used for different manipulations using the Murashige and Skoog's (MS) basal medium (Murashige and skoog., 1962) modified with various growth regulators.

Complete plant regeneration

For callus induction the Murashige and Skoog's (MS) medium was modified with 0.5mg/L 2,4-D and 0.5 mg/L BAP with 12 replicas for each of the hypocotyl explants of each variety and 8 explants per flask. All the pre- sterilized flasks were plugged with the sterilized cotton, labeled and incubated at 25±2 °C under a 16/8 h day/night photoperiodic regime (1000-Lux) for 14 days and then all the explants were transferred to shock medium. A new method of brief shock to the explants was introduced in which all the surviving and uncontaminated explants induced for callus were transferred to simple Murashige and Skoog's (MS) medium (Table 3.1) solidified with 8g/L agar without any growth regulators to provide a brief shock to the explants and kept in the growth chamber conditions for 7 days then transferred to shooting medium. The shooting medium was prepared by modifying the Murashige and Skoog's (MS) medium with 0.1mg/L NAA and 2 mg/L BAP to culture all the surviving explants from the shock media. After 21 days the explants were subcultured on the same shooting medium. The visual observations were taken on weekly basis continuously. The shoots were cut from the calli

obtained from the shooting media through a sterilized razor blade and transferred to the media having half strength MS salts with 10 mg/L sucrose, 1.2g/L agar and 0.5 mg/L IBA were used to induce rooting in the calli. The adjuvant $AgNO_3$ is a prerequisite in all brassica napus tissue culture media (Akasaka-Kennedy *et al*.,2005). The shooted calli regenerating roots thus give rise to complete plants having both shoots and roots. The number of plants producing shoots and total shoots were counted. The Callus Induction Frequency (CIF), Shoot Induction Frequency (SIF), Root Induction Frequency (RIF) and Plant Regeneration Frequency (PRF) were calculated by the formulae (Moghaieb *et al*., 2005). The Mean, Standard Error of Mean (SEM), range and Standard Deviation (S.D) for the average shoot number were confirmed by the Graphpad PRISM and SPSS Softwares. The regenerated plantlets were successfully acclimatized and transferred to glass house.

Results and discussions

In the present study media with different combinations of Auxin (2,4-D) to Cytokinin (BAP} were used to induce callus in the hypocotyl and cotyledonary explants of the *Brassica napus* L. cv Cyclone, Star and Westar. The study showed that high percentage (92-99 %) formed callus. There was no significant difference between the different explants of a cultivar and among the cultivars for callus induction ($P<0.05$).The cotyledonary explants generally gave calli with more mean weight and mean Length. The same results obtained by Zhang and Bhalla (1999). They used BAP, NAA and GA_3 in the study of seven commercial Australian cultivars of oilseed *Brassica napus* seedlings obtaining high callus induction (85-100 %). The results are in consistence with Moghaieb *et al*., (2005) who obtained high 99-100% Callus induction Frequency (CIF) using MS

salts with B5 Vitamins and 1mg/L 2,4-D later on transferred it to shooting media. According to the review of Cardoza and Stewart (2004a) the hypocotyl segments were the most desirable for plant tissue culture and hence been used for most *Brassica* species because of their ability to regenerate. Khan *et al*., (2000a) has used the upper and lower portions of hypocotyl explants. In the present study generally the cotyledonary explants showed more CIF and produced calli with more mean weight and mean Length. The findings of Stewart *et al*., (1996) as well as Cardoza and Stewart (2003) showed a lower Callus induction for Canola cultivar Westar. In the first case the main reason for the lower callus induction was hyperhydration. Hyperhydration can retard the growth of the Westar tissues which was possibly traced to occur due to the gelling agent concentration. The problem in the present study occurred but at later stages when the Westar Callus was just starting shoots initiations. Therefore this may have reduced the percentage of shoot regeneration. The hyperhydration can also occur due to high cytokinin level, high temperature and type of the culture vessel. In such stresses there is more water retention and plants take up more water. The percentage of explants forming shoots varied greatly between the *Brassica napus* L. cultivars Cyclone, Star and Westar as well as the different explants (hypocotyl and cotyledon) on MS media with the Auxin (NAA) to Cytokinin (BAP) ratio. These findings exhibited a varied response (3-34%) to shoot regeneration of different explants from the cultivars. The Shoot Regeneration Frequency (SRF) is 34% for Star hypocotyl explants and 15.5% for Star Cotyledonary explants (Table 4.4). The findings of Zhang and Bhalla (1999) show that one of the *Brassica napus* cultivars named RK-7 had a low shoot regeneration (18%) from cotyledonary explants while the same cultivar had a higher (27%) shoot regeneration from the hypocotyl explants thus it is

clearly showed that the regeneration depend on explants type and genotype. The average shoot number for the hypocotyl and cotyledonary explants of the cultivars Star, Westar and Cyclone in the present study varied from 2-5.6. Zhang and Bhalla reported a lower average shoot number range (1.13-2.55). Jain *et al*., (1988) reported average shoot number of shoots per cotyledonary explant varied from 0 to as many as 50 in some *Brassica* species. Khan *et al*., (2002b) obtained 92% regeneration for cyclone using NAA and BAP along with other adjuvants as gibberilic acid and MES (Morpholinoethane sulfonic acid) using the upper portions of the hypocotyl and 97% regeneration from the lower portion of the hypocotyl by the same method for Canola cultivar Dunkled. Khan *et al*., (2003) obtained the same results for Cyclone. The low percentage of shoot regeneration in the present study may be due to the combination of the Auxin (NAA) and Cytokinin (BAP). The results reported by Jain *et al*., (1988) show that BAP in combination with NAA yielded no or a reduced number of shoots. The Westar has a reduced Shoot Regeneration Frequency (9%) for hypocotyl explants and 3% for Cotyledonary Explants as evident from Table 4.4. Cardoza and Stewart (2003) reported 16.9 % regeneration for the cultivar Westar after a 6.4 % loss with hyperhydration but the hormonal regime was pre conditioned with 1mg/L 2,4-D followed by 4mg/L BAP and 2mg/L Zeatine. The present study showed that Shoot Regeneration Frequency (SRF) was 20% for Cyclone Cotyledonary explants while 5% for the Cyclone hypocotyl explants. The rate of regeneration was slower with the hypocotyl explants as compared to Cotyledons. The same results have beeen reported by Kehra and Mathias (1992) that the important factors for shoot regeneration were explant type and genotype and the influence of hormone regime was negligible. In the present study cyclone has a

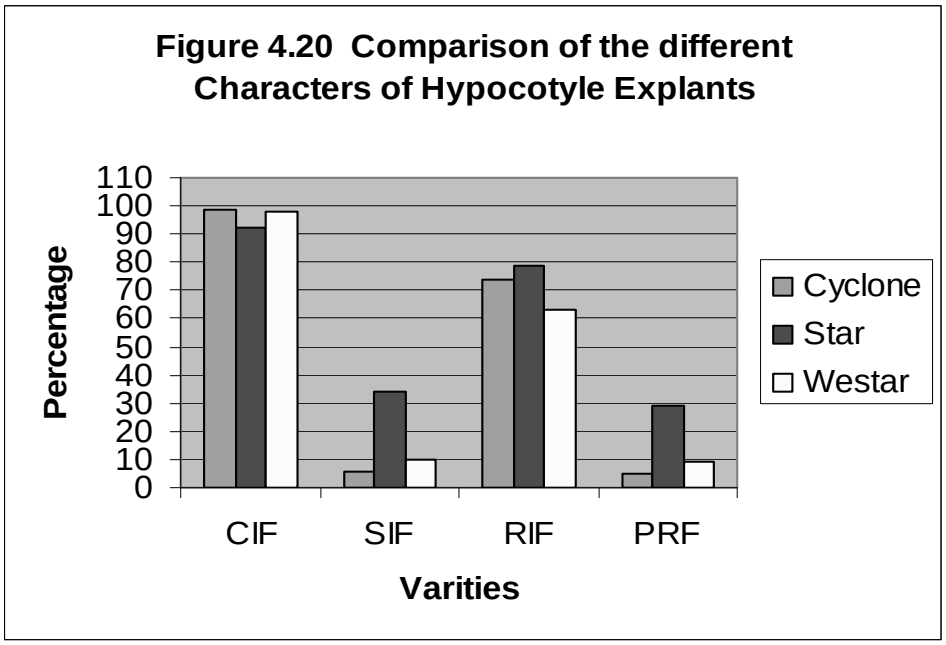
lower (5%) regeneration in hypocotyl explant as compared to the 15.5% from its Cotyledonary explants it is in consistence with Irwin *et al*., (1999). According to Phogat *et al*., (2000) *Brassica napus* cultivar GSL-1 showed better regeneration efficiency than Westar. It means that regeneration is genotype specific. According to the review by Cardoza and Stewart (2004a) genotype is a limiting factor that severely limits the germplasm that can be manipulated or improved. Regeneration also depends on the age of the explants. Young explants gave better results than older explants. In *Brassica napus* 4 day old seedling explants yielded optimal regeneration of 90% as stated by Ono *et al*., (1994). The regeneration kept on decreasing when the age of explant increased above 4days. He also reported that there was a huge variation from 0-90% in the 100cultivars tested. Jin *et al*., (2000) reported in cabbage explant of 2 week old seedling gave optimal results. Xiang *et al*., (2000) used hypocotyl explants of 5day old seedlings under different combinations of BAP (2,4,6 mg/L) and NAA (1,2,3 mg/L) with silver nitrate for *B. compestris ssp parachinensis* and found the best results at 4.0 mg/L BAP and 2.0 mg/L NAA. While explants younger or older resulted in lower shoot regeneration. Stewart *et al*., (1996) obtained shoot regeneration for cultivar Westar almost similar to the present results. The root induction (0.277%) is very low than the root induction for Westar (58%) obtained from the present study. The high root regeneration frequency may be due to the use of basal part of the hypocotyl which is also reported by Slesak *et al*., (2005) who obtained rhizogenesis for hypocotyls (98-100%) and cotyledons (54-85%) of cultured *in vitro* in *Brassica napus* L., cv. Kana. Short treatment (1 and 3 days) through MS media having 2,4-D and then transferred to hormone free medium obtaining adventitious shoots with the highest frequency (14% of explants) on hypocotyls cultured. Histological

analysis clearly indicated that the basal part of hypocotyls was involved in root formation and callus production, and the apical part for shoots. Similar method was used by Felik *et al*., (2005) who reported more calli than untreated controls when the upper apical part of the hypocotyl was facing the cathode of the electric field. The hypocotyle segments from upper part of rape (*Brassica napus* L., cv. Goczanski) hypocotyls were stimulated by different combinations of voltage/time. Based on these results they suggested that electric field action can be connected with its influence on specific concentration of oxidative substances and hormone distribution in cells. In contrast to changes in fresh weight, electric field treatment (30 V/30 s) stimulated a higher accumulation of 2,4-D and BAP in basal parts of hypocotyls than in apical ones. Damgaard and Rasmussen (1991) reported clones of hairy root formation which were sub cultured on hormone free liquid MS medium but the results are not available. Julliard et *al*., (1992) compared the regeneration abilities of the in vitro cultured explants on media supplemented with several plant growth regulator combinations. There was no regeneration on hormone free media. The method is different from the present study in the sense that hormone free medium can not be permanently used but for a brief period of seven days only.

Variety		Cyclone		Star		Westar	
Explant type		Hypo	Coty	Hypo	Coty	Hypo	Coty
Total Explant no.		80	79	94	90	92	95
Callus Inducing explants	no	79	87	87	85	90	93
	%	99	99	92.5	94.4	97.8	97.9
shoot initiations	no	5.0	20	32	14	9	3
	%	6	23	34	15.5	9.8	3.2
Roots inducing explants	no	59	60	74	80	58	57
	%	73.7	68	79	89	63	60
regenerated plants	no	4	19	27	14	8	3
	%	5	20	29	15.5	8.7	3.2
Number of shoots	*Range	1-5	3-9	1-9	1-6	1-8	2-4
	mean ± S.E.M	2 ± 1	5.6 ± 0.84	4.8 ± 0.70	3.5 ± 0.885	3.14 ± 0.857	3 ± 1
	S.D	2	2.387	2.443	2.168	2.268	1.414

Table 4.5 The Callus, Shoot, Root and Complete Plant Regeneration From Hypocotyl and Cotyledonary explants of *Brassica napus* L cv Cyclone, Star and Westar at 28th day on Shooting Media.

*Range shows minimum and maximum values
*Hypo shows Hypocotyl and coty shows cotyledon



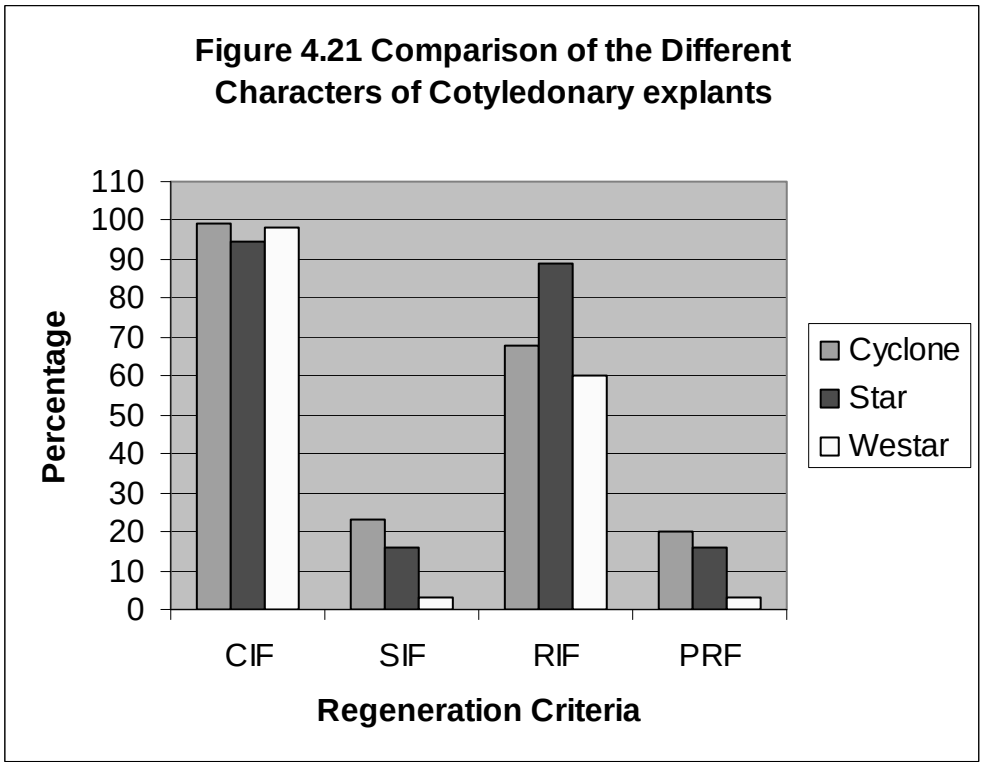


Figure 4.22 Calli of B. napus cv Cyclone, Star and Westar

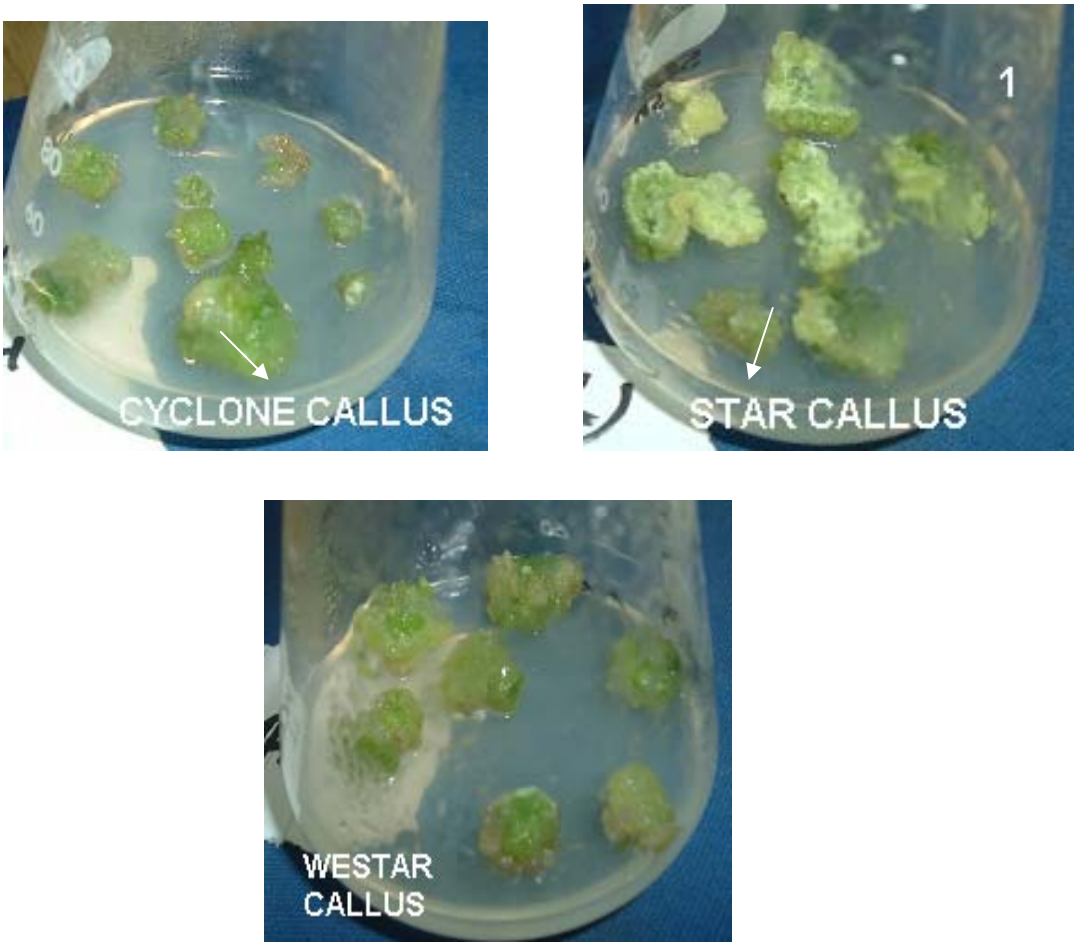


Figure 4.23 New Shoot Initiation from the Callus and Complete Regenerated Plantlets with both Shoots and Roots

Acclimatization and Transfer to Glass House

The calli having both shoots and roots were washed off the media from their roots completely and transferred in five inch plastic pots using the available potting mix and sphagnum peat moss. The pots were covered with plastic dome to retain humidity and transferred to glasshouse conditions. In the glass house the plastic dome was removed off the pots and plantlets were let to acclimatize the glass house conditions. After acclimatization the plantlets were successfully transferred to the soil.

Figure 4.25 Acclimatization and Transfer to Glass House

Recommendations

The Authors are very much thankful to the Higher education commission, Pakistan for funding the research work.

References

Akasaka-Kennedy, Y., H. Yoshida and Y. Takahata. (2005). Efficient plant regeneration from leaves of rapeseed (*Brassica napus* L.): the influence of AgNO3 and genotype.24 (11): 649-54.

Cardoza, V and C.N. Stewart. (2003). Increased Agrobacterium-mediated transformation and rooting efficiencies in canola *(Brassica napus* L.) from hypocotyl segment explants. *Plant Cell Rep.* 21: 599-604.

Cardoza, V and C.N. Stewart. (2004a). Invited review: *Brassica* biotechnology: Progress in cellular and molecular biology .in vitro. *Cell. Dev. Bio.plan.*40 (6). 542-551(10)

Cardoza, V and C.N.Stewart. (2004b). I.S. curtis (ed), Transgenic crops of the world-essential Protocols, Kluwer Academic Publishers. 379-387.

Damgaard, O and O. Rasmussen. (1991). Direct regeneration of transformed shoots in *Brassica napus* from hypocotyl infections with Agrobacterium rhizogenes. *Plant Mol Biol.*17(1):1-8.

Fay, M.F. (1992). Conservation of rare and endangered plants using *in vitro* methods. In: *Vitro Cellular and Developmental Biology*. 1-4. 28

Filek ,M., M. Holda , I. Machackova and J. Krekule. (2005). The effect of electric field on callus induction with rape hypocotyls.*pubmed*. 60(11-12):876-82.

Friedt, W and W. Lühs. (1998). Recent developments and perspectives of industrial rapeseed breeding. *Fett/Lipid* 100: 219-226.

Giddings, G., G. Allison, D. Brooks and A. Carter. (2000). Transgenic plants as factories for biopharmaceuticals. *NatureBiotechnology*, 18: 1151-1155.

Greville, L.T.D. (2005). Plants for a Future, Blagdon Cross, Ashwater, Beaworthy, Devon, EX215DF, UK. http://www.pfaf.org/cgi-in/pfaf/arr_html?Brassica+ napus+ napo*Brassica*)

Irwin, J.A., P. Sparrow and P.J. Dale. (1999). Towards anunderstanding of genotypic variation in *Brassica oleracea* transformation efficiency. *The regional institute Ltd*.

Proceedings of the 10th International rapeseed congress Canberra, Australia. http://www.regional.org.au/au/gcirc/4/38.htm

Jin, R.G., Y.B. Liu, B.E. Tabashnik, D. Borthakur. (2000). Development of transgenic cabbage (*Brassica oleraceae var. capitata*) for insect resistance by *Agrobacterium tumemaciens* mediated transformation. *In vitro Cell. Dev. Biol. Plant* 36:231-237

Julliard, J., B. Sotta, G. Pelletier, and E. Miginiac. (1992). Enhancement of Naphthaleneacetic Acid-Induced Rhizogenesis in T_L-DNA-Transformed *Brassica napus* without Significant Modification of Auxin Levels and Auxin Sensitivity. *Plant physiol*.100(3): 1277–1282.

Katavic V., W. Friesen, D.L. Barton, K.K. Gossen, E.M Giblin, J. Luciw, J. An, I.T. Zou, S.L. MacKenzie, W.A Keller, D. Males and D.C. Taylor. (2001). Improving erucic acid content in rapeseed through biotechnology: What can the Arabidopsis FAEI and the yeast SLCI-I genes contribute? *Crop Science*, 41: 739-747.

Khan, M.R., H. Rashid and A. Quraishi. (2002a). Effects of various growth regulators on callus formation and regeneration in *Brassica napus* Cv. Oscar. *Pakistan J. Biol. Sci.* 5: 693-695.

Khan, M.R., H. Rashid and A. Quraishi. (2002b). High frequency shoot regeneration from hypocotyl of canola *(Brassica napus* L.) cv. Dunkled. *Plant Tissue Cult.* 12: 131-138

Khan, M.R., H. Rashid, M. Ansar and Z. chaudry. (2003). High frequency shoot regeneration and Agrobacterium-mediated DNA transfer in Canola (*Brassica napus*). *Plant cell Tissue and org Cult.*75: 223-231

Khehra, G.S and R.J Mathias. (1992). The interaction of genotype, explant and media on the regeneration of shoots from complex explants of *Brassica* napus. *J.Exp.Bot.*43: 1413 –1418.

Lakshmanan, P., K.N. Siew, S.C. Loh and J.C. Goh. (1997). Auxin, cytokinin and ethylene differentially regulate specific developmental states associated with shoot bud morphogenesis in leaf tissues of mangosteen *(Garcinia mangostana* L.) cultured in vitro. *Plant Cell Physiol.* 1: 59 - 64.

Moghaieb, R.E.A., A.E.A. Mohamed, G. El .M. Rabab, S.Y. Sawsan and M.E.S. Ahmed (2005) A reproducible protocol for regeneration and transformation in canola *(Brassica napus L.*). *Afric. J. Biotech.* 5 (2): 143-148

Murashige, T and F. Skoog. (1962). A revised medium for rapid growth and bioassays with tobacco tissue culture. *Physiol. Plant.* 15:473 - 379.

Neeser, C., H. Bergmans, S.O. Duke, N. Ellstrand, D. Heron, R. MacDonald, M McLean, T. Nickson, M .R. Lehto, J. Schiemann, C. N. Stewart G. Tomim. (1999). Report of the *brassica* crops working group.

Ono, Y., Y. Takahata and N. Kaizuma. (1994). Effect of genotype on shoot regeneration from cotyledonary explants of rapeseed (*Brassica* napus L.). Plant Cell Rep. 14:13–17

Phogat, SK., P.K. Burma and D. Pental. (2000). High frequency regeneration of *Brassica napus* varieties and genetic transformation of stocks containing fertility restorer genes for two cytoplasmic male sterility systems. *J. Plant Biochem. Biotechol.* 9: 73-79.

Slesak, H., M. Popiearska and G. Goralski. (2005). Morphological and Histological Aspects of 2,4-D Effects on Rape Explants (*Brassica napus* L. cv. Kana) Cultured In vitro. *Acta Biologica Cracoviensia Series Botanica* 47(1): 219-226

Stewart, C.N., M.J. Adang, J.A. All, P.L. Raymer, S Ramachandran and W.A. Parrott. (1996). Insect control and dosage effects in transgenic canola containing a synthetic Bacillus thuringiensis cryIAC gene. *Plant physiol*ogy, 112: 115-120.

Thorpe, T.A., (1990). The current status of plant tissue culture. In S.S. Bhojwani(Ed.), Plant tissue culture: Application and limitations pp.1-33). Amsterdam: Elsevier.

Thrope, T.A. (1993). *In vitro* oganogenesis and somatic embryogenesis : physiological and biochemical aspects. In: (Morphogenesis in Plants). Edited By Roubelakis – Angelakis, K A and Tran Thaan Van K, Plenum Press, New York.19 –38.

Willcox, J.K., G.L. Catignani, S. Lazarus. (2003). Tomatoes and cardiovascular health. *Crit Rev Food Sci Nutr*.43(1):1-18

Xiang,Y., W.K.R. Wong, M.C. MA, R.S.C. Wong.(2000) Agroabacterium mediated transformation of *Brassica compestris* ssp. *Parachinensis* with synthetic *Bacillus thuringiensis* Cry1Ab and Cry1Ac genes. *Plant Cell Rep*. 19:251-256

Zhang, Y. and P. L. Bhalla. (1999). Shoot regeneration potential from seedling explants of Australian cultivars of oil seed rape (*Brassica napus* l.). 10th International rapeseed congress Canberra Australia.
http://www.regional.org.au/au/gcire/4/494.htm.