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Dedicated to Prof. dr. MERCEDES WRISCHER
on the occasion of her 70th birthday.

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SHOOT AND ROOT REGENERATION FROM CALLUS TISSUE OF *Allium commutatum* Guss.

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Callus tissue was induced on root tips of *in vitro* cultured seedlings of *Allium commutatum* Guss. on MS medium supplemented with 3 % sucrose, 0.8 % agar, 4.5 μM 2,4-dichlorophenoxyacetic acid (2,4-D) and 4.6 μM kinetin. Pieces of developed calli were transferred to MS medium with different concentrations of 2,4-D (0.02, 0.05 and 0.1 μM) and kinetin (0.02, 0.1 and 0.5 μM) or without them. After five weeks of cultivation, callus proliferation and differentiation as well as adventitious shoot and root regeneration were analysed. The best callus proliferation and adventitious root induction were achieved on MS medium containing 0.1 and 0.5 μM kinetin; addition of 2,4-D had no significant influence. Higher concentrations of kinetin also favoured higher incidence of meristemoids. Adventitious shoot development was noticed only on three media tested.

Key words: *Allium commutatum*, callus culture, differentiation, regeneration

Introduction

The genus *Allium* comprises up to 700 species, including some that are economically important such as *A. cepa* L., *A. sativum* L., *A. ampeloprasum* L. and *A. porrum*

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L. Some of them are important medicinal plants due to their antibiotic, antitumor and antithrombic effects on animal cells (AYABE et al. 1995), while some have frequently been used as bioassay organisms (PAVLICA et al. 1998, RANK and NIELSEN 1997).

A. commutatum Guss. is a typical Mediteranean species. So far it has been used in cytogenetic investigations of callus cultures (PAVLICA and PEVALEK-KOZLINA 1999) and karyological investigations of natural populations (BESENDORFER et al. 1997). The aim of this study was to induce callus tissue formation and to investigate the influence of 2,4-D and kinetin on regeneration ability in *A. commutatum* Guss.

Material and methods

Root tips of *in vitro* cultivated seedlings of *A. commutatum* were used as initial explants for initiation of callus culture. The seedlings were grown from seeds collected in their natural habitat (Makarska, Croatia).

The sterilisation of seeds was carried out with 2 % (w/v) water solution of the chlorine product Izosan-G (99 % sodium dichloroisocyanurate dihydrate, Pliva, Zagreb) for 5 min. After three rinses in sterile distilled water (5 min each), seeds were treated with 6 % solution of hydrogen peroxide for 5 min. After three washes in sterile distilled water (5 min each), the seeds were inoculated in test tubes filled with MS medium (MURASHIGE and SKOOG 1962) with 3 % sucrose and 0.8 % agar.

After two weeks, root tips (up to 0.3 cm long) were separated from seedlings and used as initial explants for callus induction on MS medium containing 4.5 μM 2,4-D and 4.6 μM kinetin.

Pieces of developed calli (0.5 × 0.5 × 0.5 cm) were transferred to MS medium supplemented with different concentrations of 2,4-D (0.02, 0.05 and 0.1 μM) and kinetin (0.02, 0.1 and 0.5 μM) or without them. The pH value of all media tested was adjusted to 5.8 before autoclaving at 118 kPa and 120 °C for 15 minutes. Twelve explants per each treatment were inoculated.

All cultures were incubated at 20 ± 2 °C under a 16 hour photoperiod (40 W fluorescent light, 80 $\mu E^{-2}s^{-1}$). After five weeks in culture the increase of fresh callus weight was estimated. Callus morphology as well as adventitious shoot and root regeneration were examined as well.

Samples of calli were also used for histological analysis. They were fixed in FAA (formalin, glacial acetic acid, 70 % ethanol, 1:1:18) and dehydrated in graded ethanol series (70 %, 80 %, 96 %), a mixture of ethanol and buthanol (2:1), a mixture of ethanol and buthanol (1:2) and buthanol. Afterwards, the callus was embedded in paraffin, then cut into 10-μm sections on a rotary microtom. The sections were stained with 0.05 % toluidine blue (10 seconds) and analysed under a light microscope. At least 100 sections per treatment were screened (GERLACH 1977).

Results and discussion

Seed sterilisation and germination

The sterilisation procedure was satisfactory. The percentage of sterile cultures was 92.2 %. All inoculated seeds germinated.

Callus induction

Segments of root tips (0.3 cm long) were cut off and inoculated on MS medium supplemented with 4.5 μM 2,4-D and 4.6 μM kinetin. The development of callus tissue was noticed 7–10 days after inoculation in 92.2 % of explants. After 4-weeks incubation pieces of calli were transferred to MS medium supplemented with 15 different concentrations and combinations of 2,4-D and kinetin or without them (as shown in Tab. 1).

Tab. 1. The effect of growth regulators on adventitious organ induction in callus culture of *Allium commutatum*. Basal medium: MS with 3 % sucrose and 0.8 % agar (estimated after 5 weeks in culture).

Growth regulator		Callus Tissue					
		Adventitious shoots*			Adventitious roots*		
2,4-D (μM)	Kinetin (μM)	0	<10	>10	0	<10	>10
0	0	91.7	8.3	0	50	16.7	33.3
0.02	0	100	0	0	63.6	9.1	27.3
0.05	0	100	0	0	58.4	25	16.6
0.1	0	100	0	0	58.4	33.3	8.3
0	0.02	100	0	0	66.7	25	8.3
0.02	0.02	100	0	0	25	50	25
0.05	0.02	100	0	0	58.4	8.3	33.3
0.1	0.02	91.7	8.3	0	25	8.3	66.7
0	0.1	100	0	0	33.3	0	66.7
0.02	0.1	100	0	0	0	40	60
0.05	0.1	91.7	8.3	0	0	12.5	87.5
0.1	0.1	100	0	0	9.1	27.3	63.6
0	0.5	100	0	0	33.4	8.3	58.3
0.02	0.5	100	0	0	41.6	41.7	8.3
0.05	0.5	100	0	0	50	0	50
0.1	0.5	100	0	0	16.7	50	33.3

* The data are shown as percentage of callus cultures with induced adventitious organs

Callus proliferation

Callus proliferation was relatively slow and it was strongly affected by growth regulators, especially by kinetin (Fig.1). HANSEN et al. (1995) reported typical slow growth of *A. cepa* callus also. The callus growth was better when higher concentrations of kinetin were added to the basal medium. The highest increase of callus fresh weight was observed on a medium supplemented with 0.1 μM kinetin. Callus development on MS medium with 0.02 μM or without

kinetin was very poor. Although 2,4-D was important for callus induction (YAMADA et al. 1971, NOVAK et al. 1986), it had no major influence on callus proliferation.

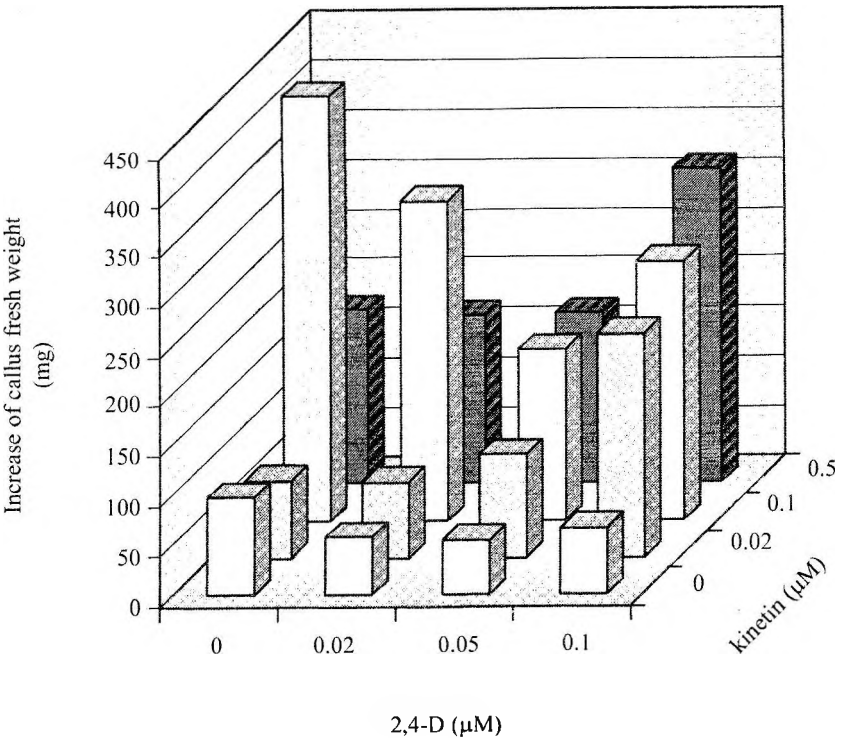
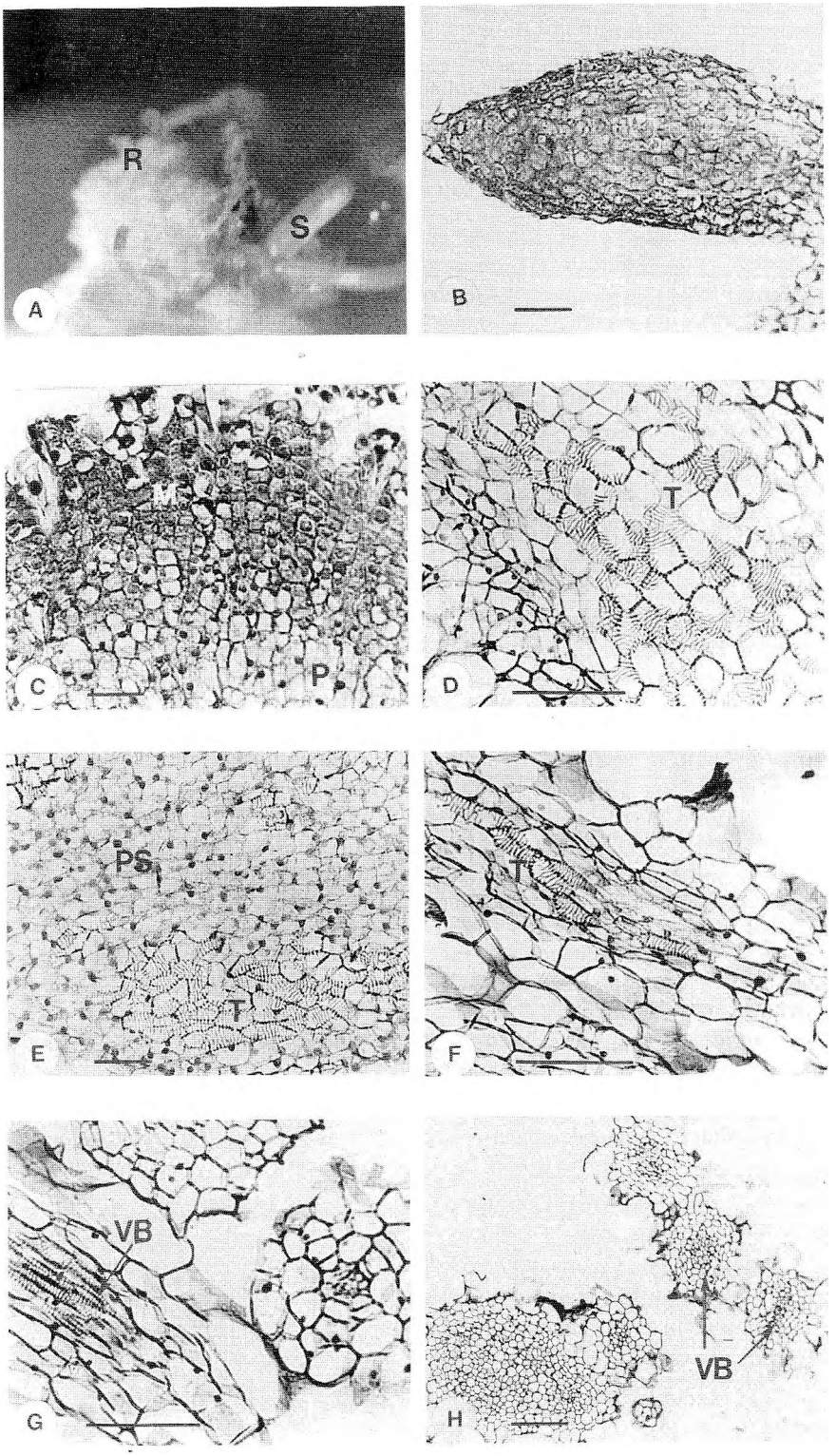


Fig. 1. Increase of callus fresh weight estimated after five weeks of cultivation on media supplemented with different concentrations and combinations of 2,4-D and kinetin or without them.

Fig. 2. **A**, Adventitious shoots and roots developed in callus tissue of *Allium commutatum* on MS medium with 0.1 μM 2,4-D and 0.02 μM kinetin; **B-H**, Sections through callus tissue cultivated on MS medium with: **B**, 0.1 μM 2,4-D and kinetin with a well developed meristem; **C-D**, 0.1 μM 2,4-D and 0.5 μM kinetin with peripherally located meristematic region and central undifferentiated parenchymal cells (**C**) and tracheidal element formation (**D**); **E**, 0.02 μM 2,4-D and kinetin with procambial strands between differentiated tracheidal elements; **F**, 0.05 μM 2,4-D with tracheidal elements arranged in rows; **G**, 0.2 μM 2,4-D with longitudinal and transversal sections through adventitious roots with primitive vascular bundles; **H**, 0.1 μM 2,4-D and 0.02 μM kinetin with transversal sections through adventitious roots with developed vascular bundles. Pictures taken after 4 weeks in culture. (Bar = 0.1 mm)

S – adventitious shoot
M – meristematic region
PS – procambial strand
VB – vascular bundles
R – adventitious root
P – parenchymatic cells
T – tracheidal elements


R
S
A
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Callus morphology

After five weeks of cultivation on all media tested, *A. commutatum* callus tissue remained predominantly yellowish, slimy, clodded and loose. The callus tissue developed on media with higher concentrations of growth regulators was more compact. Partial callus browning was noticed on the medium with 0.5 μM kinetin and 0.1 μM 2,4-D. Media with 0.02 μM kinetin or without kinetin favoured the development of whitish coloured callus tissue. Appearance of greenish coloured callus was noticed on media supplemented with 0.02 and 0.1 μM kinetin, especially when combined with 0.02 μM and 0.05 μM 2,4-D.

Shoot and root regeneration

Adventitious roots and shoots were regenerated from callus tissue cultivated on MS medium without plant growth regulators (Tab. 1, Fig. 2.A). Addition of 2,4-D in combination with 0.02 μM or without kinetin decreased the number of regenerated adventitious roots. The promotive effect of kinetin was observed when it was added in higher concentrations (0.1 and 0.5 μM). Some of adventitious roots were green in their basal part. The synthesis of chlorophyll in basal parts of roots has already been reported by FRIDBORG (1971).

Development of adventitious shoots was observed only on three media tested (Tab. 1). DUNSTAN and SHORT (1978), PHILLIPS and LUTEYN (1983) and SHAHIN and KANEKO (1986) have also reported low incidence of adventitious shoot development from callus in the genus *Allium*.

Shoot and root regeneration could be improved by supplementing the nutrient medium with N_6-(2-isopentenyl)adenine (2iP) (TANIKAWA et al. 1998) or picloram (PHILLIPS and LUTEYN 1983) as well as with some natural products like adenine, caseine hydrolyzate, coconut milk or yeast extract (AYABE et al. 1995). Anyway, one should bear in mind the significant karyological variability in callus cultures (NOVAK 1990; SEO et al 1995, 1996) before regenerating plants via the callus phase.

Histological analysis

Histological analysis of callus tissue revealed the presence of parenchymatous ground tissue on all media tested (Fig. 2.C). Some parenchymal cell clusters were surrounded with tracheidal elements.

Individual tracheidal elements (Fig. 2.D) as well as tracheidal elements arranged in rows (Fig. 2.F) could be observed on all media tested. In callus tissue cultivated on media containing 2,4 -D, tracheidal elements were arranged in procambial strands (Fig. 2.E) or vascular bundles (Figs. 2.G, H)

The formation of meristemoids (Fig. 2.B) from which adventitious roots and shoots will be differentiated was observed on MS medium supplemented with 0.1 μM kinetin. Meristematic cell regions were also found on media supplemented with 0.02 μM kinetin in combination with 0.02 μM and 0.1 μM 2,4-D.

Root primordia and well differentiated roots (Fig. 2.H) were observed in all callus tissues investigated. They were numerous on all media tested except on medium without 2,4-D and medium supplemented with 0.5 μM kinetin.

Conclusion

Root tips of 4-weeks old *Allium commutatum* Guss. seedlings grown *in vitro* were used as initial explants for callus tissue induction on MS medium supplemented with 4.5 μM 2,4-D and 4.6 μM kinetin. Callus tissue was induced on 92.2 % of explants. Developed calli were transferred to MS medium supplemented with different concentrations and combinations of 2,4-D and kinetin or without them.

Higher concentrations of kinetin (0.1 and 0.5 μM) had a stimulating effect on fresh weight increase as well as on adventitious root induction. Histological analysis showed a high incidence of meristemoids in calli grown on MS medium with higher concentrations of kinetin.

On the other hand, the addition of 2,4-D had no major influence on callus proliferation and differentiation.

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