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Dedicated to Prof. dr. sc. ZVONIMIR DEVIDÉ on the occasion of his 80th birthday

Differentiation and morphogenetic potential of *Allium commutatum* Guss. callus tissue

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Differentiation and morphogenetic potential of *Allium commutatum* Guss. callus tissue was investigated in five subcultures. Callus tissue was derived from root tips of *in vitro* grown seedlings and maintained on MS medium supplemented with 3% sucrose, 0.8% agar, 4.5 μM 2,4-D and 4.6 μM kinetin. Pieces of calli were subcultured on media with different concentrations and combinations of 2,4-D (1.0, 2.5 and 5.0 μM) and kinetin (1.0, 5.0 and 25.0 μM), or without them. After six weeks, the relative increase of callus fresh weight, morphological and histological characteristics as well as the induction of adventitious shoots and roots were investigated. Histological analysis of callui from the 1st subculture showed that callus predominantly consisted of parenchymatous cells surrounded with peripheral meristematic regions. Tracheidal elements, individual and arranged in rows, were observed in all calli investigated. The best callus growth was achieved on media containing 2.5 μM 2,4-D alone or in combination with kinetin (1.0 and 5.0 μM). Addition of kinetin to the nutrient medium stimulated adventitious shoot induction. The shoot formation was improved in later subcultures. The highest number of shoots developed on media supplemented with 5.0 μM kinetin alone or in combination with 1.0 μM 2,4-D, as well as on media with 25.0 μM kinetin alone or in combination with 1.0 or 2.5 μM 2,4-D. The induction of adventitious roots was observed during all subcultures on all media tested.

Key words: *Allium commutatum*, callus tissue, differentiation, 2,4-dichlorophenoxyacetic acid, kinetin, adventitious shoot, adventitious root

Introduction

Allium commutatum Guss. is a monocotyledonous crop species belonging to the *Liliaceae* family. It is a typical Mediterranean plant species growing in open habitats on small islands and on the coast near the sea. The species is described as diploid, 2n=2x=16, with exception of some Greek populations which are triploid and tetraploid (MATTHEW 1996). PAVLICA and PEVALEK-KOZLINA (1999) analysed cytogenetical changes in *Allium*

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commutatum callus cells. They observed relatively low level of chromosome aberrations. The low somaclonal variability makes *Allium commutatum* callus tissue appropriate for investigation of plant regeneration via organogenesis. Several authors have already reported plant regeneration from callus cultures of *Allium* species via organogenesis (DUNSTAN and SHORT 1978, SHAHIN and KANEKO 1986, SHAVEMAKER and JACOBSEN 1995, ROBLEDO-PAZ et al. 2000) and via somatic embryogenesis (SHAVEMAKER and JACOBSEN 1995, SILVERTAND et al. 1996).

The aim of this study was to investigate differentiation and morphogenetic potential of *Allium commutatum* callus tissue in five subcultures. The callus tissue was cultivated on MS nutrient medium supplemented with different concentrations and combinations of 2,4-dichlorophenoxyacetic acid (2,4-D) and kinetin as well as on medium without them.

Material and methods

Callus tissue was derived from root-tips of *in vitro* grown *Allium commutatum* Guss. seedlings (PAVLICA and PEVALEK-KOZLINA 1999) and maintained on basal MS medium (MURASHIGE and SKOOG 1962) supplemented with 0.1 g L^{-1} meso-inositol, 0.1 g L^{-1} thiamine·HCl, 0.5 mg L^{-1} pyridoxine·HCl, 0.5 mg L^{-1} nicotinic acid, 30 g L^{-1} sucrose, 8 g L^{-1} agar, 4.5 μM 2,4-D and 4.6 μM kinetin.

The pieces of calli (0.5 cm^3) were transferred to glass tubes containing 20 mL of MS medium supplemented with different concentrations and combinations of 2,4-D and kinetin, and MS medium without plant growth regulators (control medium). 2,4-D was investigated in concentrations of 1.0, 2.5 and 5.0 μM, and kinetin in concentrations of 1.0, 5.0 and 25.0 μM. The pH value of all nutrient media was adjusted to 5.8 before autoclaving.

The callus cultures were incubated in a growth chamber at 24 ± 2 °C with a 16-hour photoperiod (40 W fluorescent light, 80 $\mu E^{-2}s^{-1}$) and subcultured in a 6-week period. The relative increase of callus fresh weight, morphological and histological characteristics, as well as induction of adventitious shoots and roots during five subcultures were investigated. Samples of calli from the 1st subculture were also used for histological analysis (all methods described by VUJEVIĆ et al. 1999).

Twelve callus cultures were used for each treatment. All results were expressed as mean values ± standard deviation. Statistical significance was evaluated by Student's t-test ($P < 0.05$).

Results and discussion

Typical callus tissue of *A. commutatum* was yellowish, compact and nodular (Fig. 1). According to NOVAK (1990), all *Allium* species have a strong tendency to form a compact callus. Callus tissue was relatively slow growing, which is in accordance with the results of SEO et al. (1995). In addition to typical callus tissue, softer whitish callus was observed on media without 2,4-D. Greenish parts of calli were noticed on the majority of media tested.

The callus tissue grown on media containing 1.0, 2.5 and 5.0 μM 2,4-D and 1.0, 5.0 and 25.0 μM kinetin proliferated better than calli grown on media containing lower concentrations of 2,4-D (0.02, 0.05 and 0.1 μM) and kinetin (0.02, 0.1 and 0.5 μM) investigated by VUJEVIĆ et al. (1999). The highest increase of callus fresh weight was achieved on media

containing 2.5 μM 2,4-D alone or in combination with kinetin (1.0, 5.0 and 25.0 μM) (Tab. 1). The increase of callus fresh weight was relatively high on media containing 1.0 and 5.0 μM 2,4-D alone or in combination with kinetin,

On medium without growth regulators, fresh weight decreased. The lack of 2,4-D in nutrient media also resulted in poor callus proliferation and therefore a low increase of callus fresh weight. A similar result was reported by SEO et al. (1995), who achieved good callus formation in *Allium victorialis* var. *platyphyllum* only when cytokinin was used in combination with auxin.

We noticed a strong influence of 2,4-D on callus induction and proliferation, which is in accordance with the results of HONG and DEBERGH (1995) and CASTILLO et al. (1998). At the same time, 2,4-D has an inhibitory effect on shoot regeneration (DUNSTAN and SHORT 1978, PHILIPS and LUTEYN 1983, SHAHIN and KANEKO 1986, SILVERTAND et al. 1996), probably due to lowering cytokinin level. Decreasing the cytokinin level by auxins may occur either by inhibition of cytokinin biosynthesis or by promotion of cytokinin metabolic inactivation. The inhibitory effect of auxins could be overcome by the addition of kinetin (KAMÍNEK et al. 1997). In general, the balance between auxin and cytokinin concentration is known to be a main mechanism for *in vitro* organogenesis. This statement was postulated by SKOOG and MILLER in 1957 and reconfirmed by our research.

The addition of kinetin to the nutrient medium stimulated adventitious shoot induction (Fig. 2). The shoot formation started during the 2nd subculture. In later subcultures the number of callus cultures with adventitious shoots increased (Tab. 2). The highest number of

Tab. 1. The effect of different concentrations and combinations of 2,4-D and kinetin on *Allium commutatum* callus tissue fresh weight increase in five subcultures. Basal medium: MS with 30 g L^{-1} sucrose and 8 g L^{-1} agar (results estimated after 6 weeks in culture).

GROWTH REGULATORS (μM)		INCREASE OF RELATIVE FRESH WEIGHT *				
2,4-D	KIN	1st subculture	2nd subculture	3rd subculture	4th subculture	5th subculture
0	0	1.86 ± 2.66	1.36 ± 0.89	0.02 ± 0.09	0.16 ± 0.12	0.19 ± 0.14
1.0	0	1.83 ± 1.00	**5.66 ± 2.83**	**3.42 ± 0.70**	**3.72 ± 0.63**	**4.63 ± 1.27**
2.5	0	3.80 ± 2.50	**5.56 ± 2.83**	**3.41 ± 1.32**	**3.75 ± 1.43**	**3.29 ± 0.53**
5.0	0	0.72 ± 0.76	2.02 ± 3.20	**3.66 ± 2.26**	**2.90 ± 0.80**	**3.56 ± 0.53**
0	1.0	1.80 ± 3.27	1.65 ± 1.73	0.30 ± 0.56	0.00 ± 0.20	0.15 ± 0.10
1.0	1.0	0.79 ± 1.42	2.17 ± 1.42	**3.02 ± 1.00**	**2.40 ± 0.78**	**3.02 ± 0.65**
2.5	1.0	2.18 ± 1.31	**4.77 ± 2.70**	**3.95 ± 1.16**	**2.97 ± 0.54**	**3.49 ± 0.89**
5.0	1.0	1.59 ± 1.80	**4.69 ± 4.77**	**3.48 ± 1.08**	**2.60 ± 0.38**	**2.54 ± 0.52**
0	5.0	0.53 ± 1.34	1.55 ± 3.28	2.44 ± 2.20	1.15 ± 2.12	0.82 ± 1.08
1.0	5.0	1.16 ± 1.33	**3.88 ± 2.18**	**3.62 ± 1.01**	**3.17 ± 1.02**	**2.73 ± 0.59**
2.5	5.0	2.52 ± 1.87	**5.98 ± 1.81**	**3.39 ± 1.35**	**2.72 ± 0.80**	**2.75 ± 0.71**
5.0	5.0	1.91 ± 2.33	**6.00 ± 4.10**	**4.83 ± 1.45**	**3.63 ± 1.50**	**2.92 ± 1.15**
0	25.0	1.66 ± 1.74	2.26 ± 2.32	1.21 ± 2.01	**0.72 ± 0.71**	0.00 ± 0.34
1.0	25.0	3.85 ± 2.44	**3.46 ± 2.49**	**2.46 ± 1.74**	**2.71 ± 2.67**	**1.22 ± 1.45**
2.5	25.0	3.29 ± 3.05	**3.47 ± 1.89**	**3.50 ± 1.05**	**3.33 ± 0.86**	**3.06 ± 1.15**
5.0	25.0	1.11 ± 1.86	2.42 ± 2.16	**2.76 ± 2.48**	**3.80 ± 1.29**	2.99 ± 1.11

* mean value ± standard deviation
boldface numbers indicate significant increase ($P < 0.05$) of callus tissue fresh weight in comparison to control medium

Tab. 2. The effect of different concentrations and combinations of 2,4-D and kinetin on *Allium commutatum* callus tissue adventitious shoots induction in five subcultures. Basal medium: MS with 30 g L^{-1} sucrose and 8 g L^{-1} agar (results estimated after 6 weeks in culture).

GROWTH REGULATORS (μM)		ADVENTITIOUS SHOOT INDUCTION*				
2,4-D	KIN	1st subculture	2nd subculture	3rd subculture	4th subculture	5th subculture
0	0			2/3.0	2/7.0	
1.0	0					
2.5	0					
5.0	0					
0	1.0					
1.0	1.0					
2.5	1.0					
5.0	1.0					
0	5.0		2/11.0	9/10.7	10/8.1	8/9.3
1.0	5.0			3/3.0	4/3.3	4/1.8
2.5	5.0					
5.0	5.0					
0	25.0		3/4.3	2/8.0	2/2.0	
1.0	25.0		2/1.5	3/3.0	2/10.0	2/9.0
2.5	25.0		2/2.0	5/3.6	9/3.1	8/5.1
5.0	25.0					

* number of callus cultures with adventitious shoots / mean number of shoots per callus

shoots developed on medium supplemented with 5.0 μM kinetin alone and on medium with 25.0 μM kinetin alone or in combination with 2.5 μM 2,4-D. Adventitious shoots also developed on medium with 5.0 μM kinetin and 1.0 μM 2,4-D and on medium with 25.0 μM kinetin alone or in combination with 1.0 μM 2,4-D, as well as on control medium.

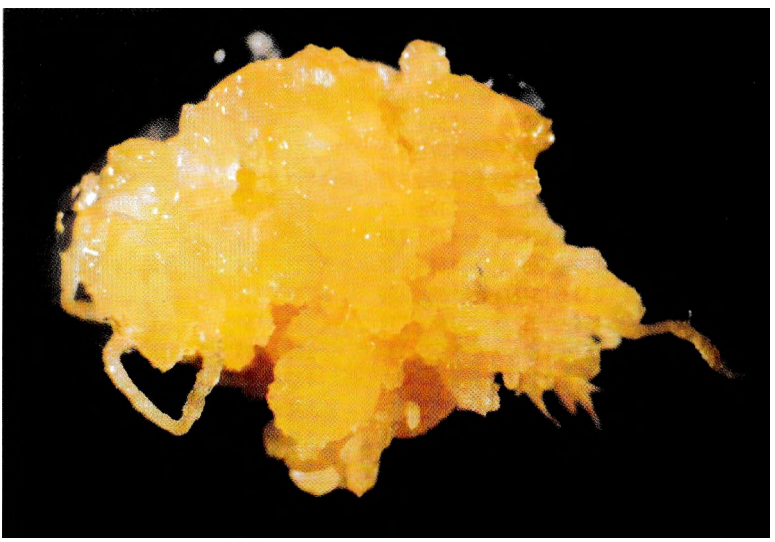

Fig. 1. Typical *Allium commutatum* callus tissue was yellowish, compact and nodular (MS medium without growth regulators).

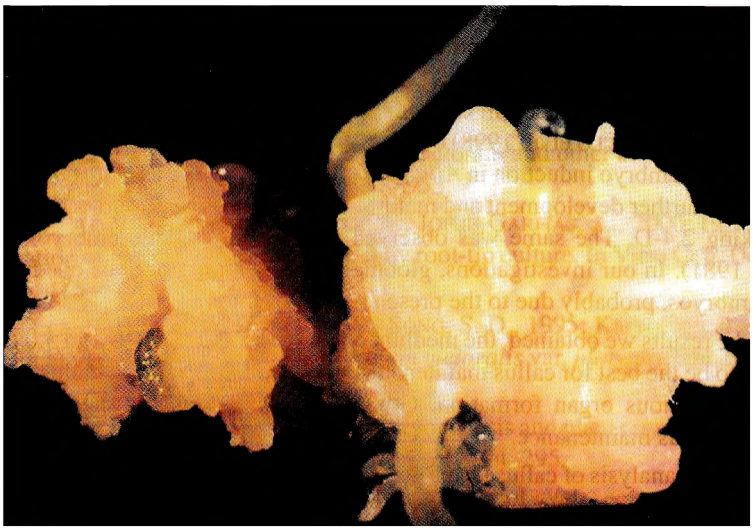

Fig. 2. Adventitious shoots induced in *Allium commutatum* callus tissue grown on MS medium supplemented with 2.5 μM 2,4-D and 25.0 μM kinetin during 5th subculture.

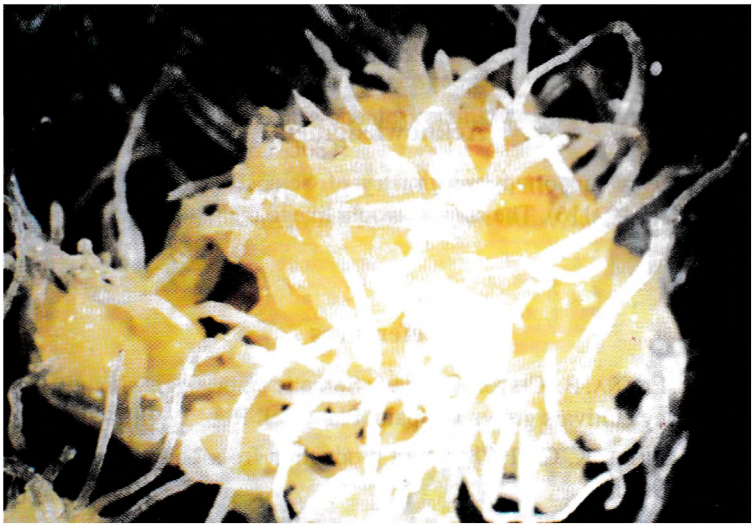

Fig. 3. Adventitious roots induced in *Allium commutatum* callus tissue grown on MS medium supplemented with 5.0 μM 2,4-D and 1.0 μM kinetin during 5th subculture.

The induction of adventitious roots was noticed in a very high percentage of callus cultures grown on all media tested during all subcultures (Fig. 3). The highest root induction potential was noticed on control medium, where more than 10 roots per callus were formed. The addition of kinetin in concentrations higher than 1.0 μM slightly inhibited the root formation potential. In callus cultures grown on media containing 5.0 and 25.0 μM kinetin, less than 10 roots per callus were developed.

The formation of globular structures resembling somatic embryos was noticed on media containing 2,4-D (in all concentrations tested) in combination with 1.0 and 5.0 μM kinetin as well as without kinetin. PAVLICA and PEVALEK-KOZLINA (1999) reported somatic embryo formation on MS medium containing 2.5 μM 2,4-D. Several authors (HONG and DEBERGH 1995, CASTILLO et al. 1998) observed that the presence of 2,4-D was a prerequisite for somatic embryo induction in *Allium porrum* L. and *Allium fistulosum* L. They also observed that further development and maturation of proembryos could occur only on medium lacking 2,4-D. The same was observed with carrot somatic embryos (SUNG and OKIMOTO 1981). In our investigations, globular structures did not developed into mature somatic embryos, probably due to the presence of 2,4-D.

From the results we obtained, the medium containing 5.0 μM 2,4-D and 5.0 μM kinetin turned out to be the best for callus maintenenace, since it allowed good callus proliferation without adventitious organ formation. GOLCZYK (1994) used this medium for *Allium sibiricum* L callus maintenance.

Histological analysis of callus tissue grown on all media tested showed that it predominantly consisted of parenchymatous cells. The clumps of parenchymatous cells were surrounded with meristematic regions. The formation of meristematic regions was vigorous in calli grown on media containing higher concentrations of kinetin (KAMÍNEK et al. 1997). Tracheidal elements, individual and arranged in rows, were observed in calli grown on all media tested. The histological analysis did not show a significant difference to results obtained by VUJEVIĆ et al. (1999), who investigated the histology of *Allium commutatum* callus tissue grown on media containing lower concentrations of 2,4-D and kinetin.

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