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Use of direct somatic organogenesis for *Agrobacterium*-mediated transformation of onion

Uporaba neposredne somatske organogeneze za transformacijo čebule s pomočjo *A. tumefaciens*

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Abstract. An *Agrobacterium tumefaciens* mediated DNA delivery is being developed for onion (*Allium cepa* L.) using organogenic structures formed on the ovaries as target tissue. An indirect transformation procedure using *A. tumefaciens* strains LBA4404 and EHA105 in combination with four plasmids was used for establishing an efficient transformation protocol. Studies were focused on different protocols for co-cultivation, selection and detection of optimal reporter and selection genes in onion. A histochemical GUS test and visual detection of the GFP protein were used for optimization of transformation treatments. Several pretreatment and co-cultivation protocols achieving transient expression were studied, and the optimal was a combination of sonication (10 s) and vacuum treatment (5 min, 35 mmHg). Among several selection media studied, optimal results were obtained using phosphinotricin (2.5 mg/l) as selection agent and timentin (150 mg/l) for good suppression of bacterial growth. The transgenic nature of individual regenerants after six months on selection media was confirmed by polymerase chain reaction using primers for detection of *uidA* and *bar* genes. Using this test, three regenerants were positive for both *bar* and *uidA* genes and three regenerants were positive for the *bar* gene only.

Keywords: Tissue culture, genetic transformation, onion, *Allium cepa* L., direct somatic organogenesis, *Agrobacterium*, *uidA*, *gfp*, *bar*, PCR

Izvleček. Organogene strukture čebule (*Allium cepa* L.) nastale z direktno somatsko organogenezo smo uporabili za razvoj uspešne metode genske transformacije pri čebuli. Organogene strukture smo okuževali s sevoma LBA4404 in EHA105 bakterije *Agrobacterium tumefaciens* in štirimi plazmidi. Proučevali smo različne pogoje kokultivacije, selekcije in izbirali primerne testne gene. Na podlagi GUS testa in z opazovanjem zelene flourescence GFP proteina z mikroskopom smo lahko optimizirali postopke predtretiranja in kokultivacije. Postopki predtretiranja so vključevali uporabo ultrazvoka (optimalno 10 s) in dveh nivojev vakuuma (optimalen 5 min pri 35 mmHg). Med mnogimi preizkušenimi selekcijskimi gojišči smo najboljše rezultate dobili pri kombinaciji selekcijskega agensa fosfinotricina (2,5 mg/l) ter antibiotika timentina (150 mg/l) za učinkovito odstranitev bakterije.

Vključitev plazmidne DNA v genom čebule smo preverili s polimerazno verižno reakcijo za gena *uidA* in *bar*. S tem testom smo pri treh regenerantih čebule po šestih mesecih na selekcijskem gojišču dokazali prisotnost *uidA* in *bar* gena, pri drugih treh regenerantih čebule pa je prišlo do vključitve samo slednjega.

Ključne besede: Tkivne kulture, genska transformacija, čebula, *Allium cepa* L., direktna somatska organogeneza, *Agrobacterium*, *uidA*, *gfp*, *bar*, PCR

Introduction

Onions and shallots (*A. cepa* L.), garlic (*A. sativum* L.) and leek (*A. porrum* L.) are important vegetable crops which have considerable health benefits. By manipulating agronomic and quality trades in this crops it may be possible to reduce the requirement for pesticide applications as it has been achieved in other crops (Phipps & Park 2002) and improve their sustainable production and nutritional status. One possible approach to this is by genetic modification. An *A. tumefaciens* DNA delivery has been developed for onion using organogenic structures formed on ovaries according to an established somatic regeneration protocol (Luthar & Bohanec 1999). The method differs from published protocols (Eady & al. 2000, Zheng 2000) which have used embryo-derived callus tissue as target cells. Our method for direct organogenesis in onion resulting in the formation of multiple shoot structures of cultured flower buds or ovaries was used as starting tissue. *A. tumefaciens* strains LBA4404 and EHA105 in combination with four plasmids were tested. Studies focused on different protocols for co-cultivation, selection and detection of optimal reporter genes for onion. A histochemical GUS test and visual detection of the GFP protein were used to optimize transformation treatments.

Material and methods

Immature flower buds were collected from five different cultivars (Belokranjka and US inbred lines B1828A, B1828B, MSU2399B and B2371 x B2923B) grown in the greenhouse. *A. tumefaciens* strains LBA4404 and EHA105 containing vectors pBIN m-gfp5-ER and pCAMBIA 1301, 1303 and 3301 were grown to log phase on liquid LB media. Ovaries with embryogenic structures were transferred into 15 ml of *Agrobacterium* suspension and exposed to sonication treatment and/or a vacuum-assisted transformation procedure. The embryogenic structures were strained on sterile filter paper and put on media containing 100 mM acetosyringone. After three days of co-cultivation, explants were transferred to BDS media containing agents for selection of transformants and prevention of *Agrobacterium* growth. The efficiency of the antibiotics, geneticin (30-50 mg/l), kanamycin (50 mg/l), hygromycin (25 mg/l) and the herbicide phosphinotricin (2.5 mg/l) as selective agents in onion tissue culture was investigated. Certain antibiotics, cefotaxim, vankomycin (250 mg/l) and timentin (150 mg/l) were used to eliminate *Agrobacterium* from plant tissue without severely inhibiting plant growth. Tissue cultures were subcultured on fresh media every three weeks. Histochemical GUS assays were performed 3-6 days after transfer of explants to the media containing selection antibiotics following the protocol of Rueb & Hansgens (1989). For GFP expression, tissues were examined by observation under a fluorescence microscope using 460-500 nm excitation and 515-560 nm barrier filters.

Results and discussion

The average formation of organogenic structures on ovaries of the five cultivars on BDSi media was 34.8%. The efficiency of the protocol for the induction of direct somatic embryogenesis on ovaries

is comparable to the results obtained by LUTHAR & BOHANEC (1999). Transient expression of GUS product was present in *in vitro* onion cultures following an *A. tumefaciens*-mediated transformation procedure. Blue histochemical staining was visible on the surface area of explants. The dark blue spots on the forming shoots were particularly promising. After three days of co-cultivation, single cells expressing m-gfp5-ER could be observed, but the expression was not mainly localized on the embryogenic cells. Several pretreatment and co-cultivation protocols achieving transient expression were studied (Tab. 1), the optimal one was a combination of sonication (10 s) and vacuum treatment (5 min, 35 mmHg) using the *A. tumefaciens* LBA4404 (pCAMBIA 3301). The mechanical treatment of ultrasound causing loosening of cell walls in the organogenic structures formed on ovaries enhanced the efficiency of *Agrobacterium* infection. SANTAREM & al. (1998) established that sonication included in the co-cultivation treatment has a major impact on the transformation efficiency of young soybean leaves. EADY & LISTER (1998) reported a beneficial effect of vacuum infiltration subsided by fine stirring of onion embryos in *Agrobacterium* suspension. Strong inhibition of *Agrobacterium* in cultured tissues was obtained with timentin at 150 mg/l. Selection media containing geneticin at 30 mg/l had a deleterious effect on regenerating tissues, hygromicin at 25 mg/l was lethal, causing excessive tissue damage after 6 weeks of exposure. Nine point eight percent of regenerants of Belokranjka sustained selection on phosphinotricin (2.5 mg/l) after six months of cultivation.

Table 1: Summary of different transformation experiments with *A. tumefaciens* LBA4404 and EHA105 (pCAMBIA1303, 1301, 3301)

Preglednica 1: Povzetek različnih poskusov transformacij s pomočjo *A. tumefaciens* LBA4404 in EHA105 (pCAMBIA1303, 1301, 3301)

EXPERIMENT / PLASMID	NO. OF ORGANOGENIC OVARIES		TREATMENT	
	A. tumefaciens LBA4404	*A. tumefaciens* EHA105	SONICATION	VAKUUM
1. / pCAMBIA 1303	3240	780	5 s	/
	270	120	/	30 min
	150	150	5 s	5 min
	120	120	5 s	30 min
2. / pCAMBIA 1301	350	270	5 s	/
	210	/	6 s	5 min
	200	/	15 s	5 min
	200	/	30 s	5 min
3. / pCAMBIA 3301	420	210	5 s	/
	200	/	5 s	5 min
	240	/	6 s	5 min
	240	/	10 s	5 min
	440	/	15 s	5 min
	240	/	20 s	5 min
	200	/	30 s	5 min
	120	/	50 s	5 min
	/	150	/	30 min
ORGANOGENIC OVARIES/EXP.	6840	1800		

The transgenic nature of individual plants was confirmed by polymerase chain reaction using primers for *uidA* and *bar* genes. PCR on three regenerants transformed with *A. tumefaciens* LBA4404 (pCAMBIA 3301) confirmed integration of *uidA* and *bar* genes into the onion genome when a combination of sonication and vacuum subsided transformation was used (Fig. 1). Using this analysis, three regenerants were positive for the *bar* gene only (Fig. 2). The survival of three regenerants on the selection media and the positive results of analysis performed by PCR show integration of only a fragment of pCAMBIA 3301 plasmid into the onion genome. Similar results were reported in an apple cultivar by YAO & al. (1995) and in rice by HIEI & al. (1997). Studies of the transformation efficiency in *Solanum muricatum* (ATKINSON & GARDNER 1991) revealed that up to 50% of transformed plants have only partial incorporation of T-DNA in the plant genome.

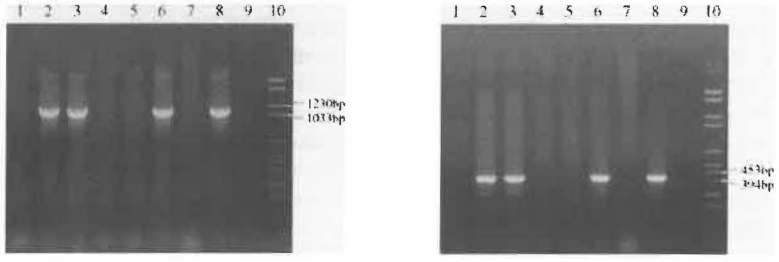

Figure 1: PCR amplification of genomic DNA from transformants with (A) *uidA* and (B) *bar* gene primers. Lanes 1, 4 and 5: untransformed regenerants, 2, 3 and 6: individual transgenic plants, 7: untreated plant, 8: pCAMBIA 3301, 9: water and 10: size leader no. 6

Slika 1: Namnoženi fragmenti DNA s parom začetnih oligonukleotidov za (A) *uidA* in (B) *bar* gen pri regenerantih čebule po okužbi z *A. tumefaciens*. 1, 4 in 5: netransformirani regeneranti, 2, 3 in 6: transformirani regeneranti, 7: kontrola - neokuženi regeneranti, 8: pCAMBIA 3301, 9: slepi vzorec in 10: velikostni standard št. 6

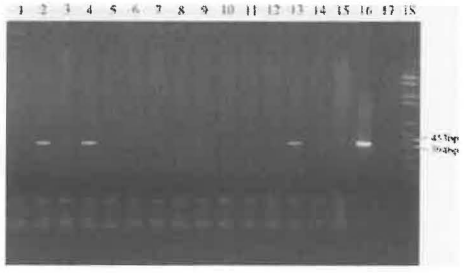

Figure 2: PCR amplification of genomic DNA from transformants with *bar* selection gene primers. Lanes 1, 3, 5 –12 and 14: untransformed regenerants, 2, 4 and 13: individual transgenics positive for the *bar* gene, 15: untreated plant, 16: pCAMBIA3301, 17: water and lane 18: size leader no. 6

Slika 2: Namnoženi fragmenti DNA s parom začetnih oligonukleotidov za selekcijski *bar* gen. 1, 3, 5 –12 in 14: netransformirani regeneranti, 2, 4 in 13: transformirani regeneranti, 15: kontrola – neokuženi regeneranti, 16: pCAMBIA 3301, 17: slepi vzorec in 18: velikostni standard št. 6

Conclusions

On the basis of results obtained it was concluded that our protocol used for the transformation of onion with *A. tumefaciens* is suitable. The transgenic nature of six individual regenerants after six months on selection media was confirmed by PCR. Further analyses of their progeny are underway. Using this test, three regenerants were positive for both *bar* and *uidA* genes and three regenerants were positive for the *bar* gene only. Transformation efficiency might be further enhanced by the application of more virulent strains of *A. tumefaciens*, specialized for monocotyledonous species (LBA4404, EHA105), a combination with different constructs could enhance the incorporation of transgenes into the target tissue, and the combination of sonication treatment and a vacuum-subsided transformation procedure could also contribute to the more frequent and stable incorporation of the desired genes.

Povzetek

Organogene strukture čebule nastale z direktno somatsko organogenezo smo uporabili za razvoj uspešne metode genske transformacije pri čebuli. Vključitev plazmidne DNA v genom čebule smo preverili s polimerazno verižno reakcijo za gena *uidA* in *bar*. S tem testom smo dokazali prisotnost obeh genov v treh regenerantih in samo *bar* gena v drugih treh regenerantih čebule. Učinkovitost transformacije bi lahko v prihodnje optimizirali z uporabo virulentnejših sevov *A. tumefaciens* primernih za enokaličnice (LBA4404, EHA105) ter ustreznih kombinacij sevov in plazmidov. Za nadaljno optimizacijo učinkovitosti stabilne vključitve željenih genov v genom čebule bi lahko med ko-kultivacijo bakterijskega in rastlinskega materiala uporabili kombinacijo ultrazvoka in vakuuma.

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Mycorrhizal potential of two forest research plots with respect to reduction of the emissions from the Thermal Power Plant Šoštanj

Mikorizni potencial dveh gozdnih raziskovalnih ploskev po zmanjšanju emisij iz Termoelektrarne Šoštanj

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Abstract. The mycorrhizal potential of two differently polluted forest research plots was determined in the emission area of the Thermal Power Plant Šoštanj. Zavodnje is the polluted, while Mislinja is the less polluted plot. Mycorrhizal potential of the soils from the two sites was estimated in a pot experiment. Types of ectomycorrhizae were identified in the soil cores and on short roots of Norway spruce seedlings. The fresh weight of needles and stems, number of short roots and the percentage of mycorrhizal short roots on seedlings from Zavodnje were significantly lower in comparison with Mislinja. The results indicate that the mycorrhizal potential of the more polluted site is lower. Mycorrhizal potential is discussed with respect to the results from our earlier studies.

Key words: ectomycorrhizae, mycorrhizal potential, Norway spruce seedlings, forest research plots

Izvleček. Določili smo mikorizni potencial dveh različno onesnaženih gozdnih ploskev v imisijskem območju Termoelektrarne Šoštanj. Zavodnje predstavlja onesnaženo, Mislinja pa manj onesnaženo raziskovalno ploskev. Mikorizni potencial obeh območij smo določili z lončnim poskusom. Tipe ektomikorize smo identificirali v talnih vzorcih raziskovalnih ploskev in na kratkih koreninah semenk smreke. Sveže teže iglic in stebel semenk (nadzemni del), število kratkih korenin in število nemikoriznih kratkih korenin semenk, ki so rastle na substratu iz Zavodenj, je statistično značilno manjše v primerjavi Mislinjo. Rezultati kažejo, da je mikorizni potencial bolj onesnaženega območja nižji. Mikorizni potencial smo primerjali z rezultati naših prejšnjih raziskav.

Ključne besede: ektomikoriza, mikorizni potencial, semenke smreke, gozdna raziskovalna ploskev

Introduction

Ectomycorrhiza is the site of exchange of nutrients between the plant and the fungus. Fungal hyphae exploit the soil for mobilisation and absorption of water and nutrients (BRUNNER 2001). They are integral, functional parts of plant roots, in which the fungi involved provide a direct link between the soil and the roots (LEYVAL & al. 1997). Furthermore the mycelia of ectomycorrhizal fungi act as temporal and spatial connections between different species of trees in the forest ecosystem (AMARANTHUS & PERRY 1994).

In the last decades damages of forest trees and ecosystems have been monitored in North America and Europe. These can be connected with the disturbances in the ectomycorrhizal symbiosis. The deposition of pollutants into the forest ecosystems leads to the acidification and/or eutrophication of the forest soil and can consequently affect the health and vitality of forest trees (BRUNNER 2001). The impact of pollution on forest soils can be estimated by determination of mycorrhizal potential of forest soil of differently polluted areas. Pollution can influence the below – ground diversity of ectomycorrhizal fungi since some types can better survive different stress factors than others (GIANINAZZI-PEARSON 1984, VODNIK & al. 1995, TAYLOR 1995).

In Slovenia the mycobioindication method for determination of pollution stress has been used (KRAIGHER & al. 1996) and in spruce forest the reduction of biodiversity of types of ectomycorrhizae due to pollution was established (KRAIGHER 1999). On the other hand biodiversity indexes were high in all beech forest research plots, therefore the impacts of pollution on beech ectomycorrhizae was not stated (AL SAYEGH PETKOVŠEK & KRAIGHER 2000). In studies of oak decline KOVACS reported that the ectomycorrhizal diversity decreased slowly but significantly in two oak stands in the north-east of Austria and the presence of some morphotypes were highly correlated with the crown-status of the trees (KOVACS & al. 2000).

The objectives of the present study were to determine mycorrhizal potential of two differently polluted forest research plots and to identify the types of ectomycorrhizae on short roots of mature trees and seedlings according to the concept of mycorrhizal succession (DIGHTON & MASON 1985, LAST & al. 1987) and biodiversity studies (KRAIGHER 1999, KOVACS & al. 2000, FERRIS & al. 2000).

Material And Methods

The mycorrhizal potential of forest soil is defined as the capability of propagules of naturally occurring fungi in forest soils to colonize roots of spruce seedlings. It is expressed as the percentage of mycorrhizal short roots of the total number of short roots in the sample (KROPAČEK & al. 1989). A modified method of a pot experiment for determination of the mycorrhizal potential was used (AL SAYEGH PETKOVŠEK 1997). Differently polluted research plots are situated in the emission area of the Thermal Power Plant Šoštanj (TPP) where the negative impact of pollution is well demonstrated in all parts of environment (SVETINA 1999, POKORNY 2000, KUGONIČ & STROPNIK 2001, RIBARIČ LASNIK & al. 2001, POKORNY & RIBARIČ LASNIK 2002). The two plots (850 m a.s.l., distric cambisol, *Luzulo-Fagetum*, predominant tree species *Picea abies*), were as similar regarding site characteristics as it was possible to select, but polluted differently by the emissions from the TPP, as indicated by the lichenological studies (BATIČ & KRALJ 1990) and by the total S% and Pb content in soil (Tab. 1, sampling done in August 2001). Soils from both plots were dug from the upper 20 cm, sieved (2 mm sieve) and used as planting substrates. At the same time types of ectomycorrhizae were identified in soil cores (275 ml volume, 0 – 18 cm deep) from both research plots. 5 seedlings (at 3 weeks of age) per pot and 5 pots per soil source were grown for six months in the germination cabinet, where the growth conditions were the same for all seedlings. After six months the seedlings were weighed, short roots were counted and the percentage of

mycorrhizal short roots and the types of ectomycorrhizae were determined. The determination of types of ectomycorrhizae followed the procedure from the "Colour Atlas of Ectomycorrhizae" (AGERER 1987-1999) and other primary sources of identification.

Data from the study in 1993 and 2002 were compared. The difference was in the reduction of the emissions from TPP: the reduction of the emissions from the Thermal Power Plant Šoštanj was from 86.147 t SO_2 in the year 1993 to 22.871 t in the year 2002. *Statistica for Windows 5.5* has been used for statistical analyses. The results represented in Graph 1 are the average values for seedlings from two different locations, the significance of the difference was evaluated by non-parametric statistical analyses (Man-Whitney U test).

Table 1: Sieved soil analysis data for two forest research plots (P93-polluted plot in 1993; P02-polluted plot in 2002; U93-unpolluted plot in 1993, U02-unpolluted plot in 2002)

	pH $CaCl_2$	pH H_2O	C%	N%	C/N	K Ekv	P Ekv	S%	Pb mg/kg	Cd mg/kg	Hg mg/kg	As mg/kg
P 93	3,76	4,3	6,5	0,30	22	0,14	Traces	0.07	-	-	-	-
P 02	3.44	4,0	16,8	0,59	29	0,10	Traces	0,09	115	0,57	0,26	6,15
U 93	3,36	3.8	17,0	0,82	21	0,50	Traces	0.06	-	-	-	-
U 02	3.99	4,6	7,47	0,34	21	0,10	Traces	0,06	53,4	0,26	0,29	6,15

Results and Discussion

The percentage of mycorrhizal short roots of seedlings from Zavodnje was significantly lower ($p<0.05$) in comparison with Mislinja (Fig. 1), consequently it could be concluded that mycorrhizal potential of the more polluted area is lower. Also other growth parameters of the seedlings were different: the fresh weights of stems and needles were higher in Mislinja, while the number of nonmycorrhizal roots were lower in Mislinja in comparison to Zavodnje. The average fresh weight of roots did not differ significantly. This is comparable also to the results of the study done eight years ago (Fig. 2, data on 1993 calculated from AL SAYEGH PETKOVŠEK 1997).

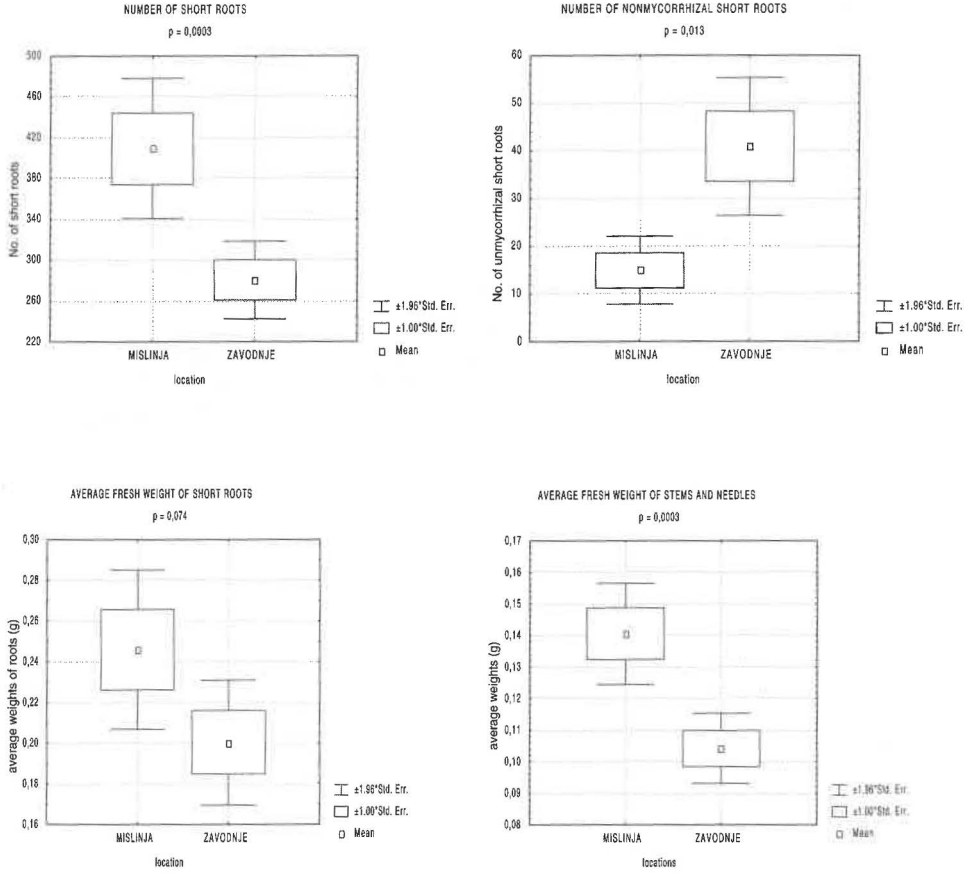

Figure 1: Comparison between different parameters of Norway spruce seedlings grown in soil substrates Mislinja and Zavodnje.

Our recent and previous study (AL SAYEGH PETKOVŠEK & al. 1995, AL SAYEGH PETKOVŠEK 1997, AL SAYEGH PETKOVŠEK & KRAIGHER 1998) on the same topic strongly indicate that pollution influences the mycorrhizal potential of forest soils. Negative impact is still present in spite of the reduction of the emissions. However the decrease in pollution seems to result in higher percentage of mycorrhizal short roots in current study as compared to former research. This increase in mycorrhization is characteristic for both plots.

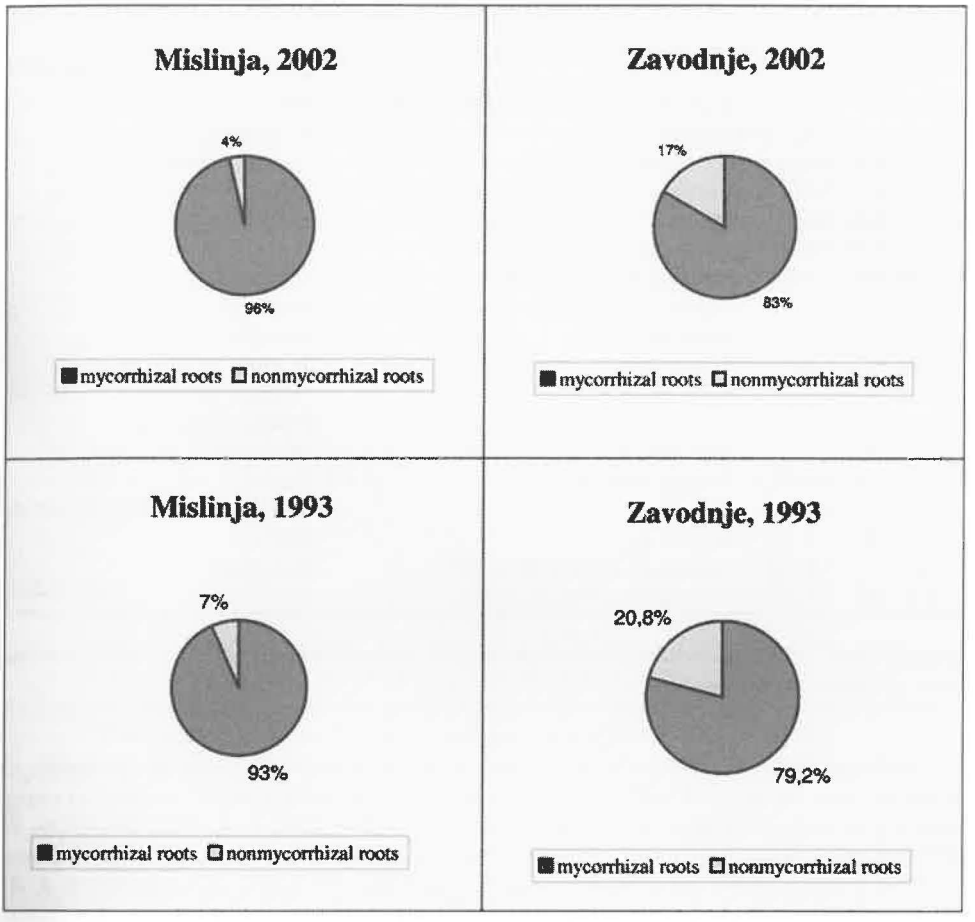

Figure 2: Mycorrhization of short roots of Norway spruce seedlings in pot experiments in the year 2002 and 1993.

Different types and different number of ectomycorrhizae were determined on short roots of mature trees in differently polluted forest research plots. 13 types of ectomycorrhizae in the soils from Mislinja and 8 types of ectomycorrhizae in the sois from Zavodnje (Tab.2) were identified. Three types of ectomycorrhizae were identical for both plots: *Cenoccocum geophilum* Fr., *Lactarius pallidus* Pers. ex Fr. and *Suillus* sp. These types belong to the group of types of ectomycorrhizae named "late stage fungi" (LAST & al. 1987). Among the type described, *Scleroderma citrinum* Pers. has been regarded as un-sensitive to pollution (ARNOLDS 1991). Also in our study *S. citrinum* was frequent in the soil samples from the polluted area (Zavodnje).

Table 2: Types of ectomycorrhizae identified in the soil cores.

TYPES OF ECTOMYCORRHIZAE		FOREST RESEARCH PLOT
SLO 801 - SA1	*Xerocomus badius* (Fr.) Kühn.: Gilbert	Mislinja
SLO 802 - SA2	*Piceirhiza obscura*	Mislinja
SLO 803 - SA3	*Cenoccocum geophilum* Fr.	Mislinja, Zavodnje
SLO 811 - SA11	*Tylospora fibrillosa* (Burt) Donk	Zavodnje
SLO 813 - SA13	*Russula acrifolia* Romagn.	Mislinja
SLO 836 - SA24	*Dermocybe sp.*	Mislinja
SLO 863 - SA63	*Thelephora terrestris* Pers.	Mislinja
SLO 875 - SA75	*Amphinema byssoides* (Pers.) J. Erikss.	Zavodnje
SLO 888 - SA70	*Xerocomus sp.*	Zavodnje
SLO 897 - SA97	*Russula sp.*	Mislinja
SLO 899 - SA99	*Tuber rufum* *	Mislinja
SLO 890 - SA52	*Lactarius pallidus* Pers. ex Fr.*	Mislinja, Zavodnje
SLO 901 - SA101	*Tuber borchii* Vitt.*	Mislinja
SLO 902 - SA102	*Boletus edulis* Bull.: Fr.*	Mislinja
SLO 903 - SA103	*Suillus sp.*	Mislinja, Zavodnje
SLO 904 - SA104	*Inocybe sp.*	Mislinja
SLO 906 - SA106	*Lactarius subdulcis* Bull.: Fr.*	Zavodnje
SLO 907 - SA107	*Scleroderma citrinum* Pers.*	Zavodnje

Legend: Types marked with * are similar (not identical or described on a different plant sp.) to the types presented in the table.

Two types of ectomycorrhizae were determined on six months old Norway spruce seedlings: *Hebeloma mesophaeum* Quél. and *Cenoccocum geophilum*. The first type is very common on young trees and could be considered as an "early stage fungus" (INGLEBY & al. 1990, DIGHTON & MASON 1985), while the *C. geophilum* has a world-wide distribution on a wide range of plants (SMITH & READ 1997) and it has been found on seedlings and mature trees (INGLEBY & al. 1990). Since black types of ectomycorrhizae named as *C. geophilum* were not well differentiated after anatomical characteristics in the past (therefore it was also considered as a "group species" as in AL SAYEGH PETKOVŠEK & KRAIGHER 1999) its different ecology might possibly been due to an involvement of several species with similar characteristics in different physiological and ecological references.

Conclusions

(i) Mycorrhizal potential as a bioassay for soil substrate pollution (with S and heavy metals) is an acceptable mycobioindication method.

(ii) The differences between the two sites were statistically significant, although the pollution effects are not highly destructive with respect to mycorrhizal soil inoculum potential.

(iii) Eventhough the emissions from the TPP were reduced in the last decade, the revitalization of soil substrates is a slow process, therefore it might take several more decades before the mycorrhizal potential of the polluted and the unpolluted site reach the same level.

(iv) Types of ectomycorrhizae present in the soils from the two sites represent all-stage as well as late-stage fungi, reflecting that soil substrates originate from old-growth forests and closed Norway spruce stands.

(v) Types of ectomycorrhizae are of primary importance for the functioning of the forest ecosystem, however the simplified situation in the pot studies of mycorrhizal potential supports the primary importance of the % of mycorrhizal short roots while identification of types of ectomycorrhizae is of a secondary importance for application in bioindication methods.

(vi) Due to the still noticeable impact of pollution on mycorrhizal potential of forest soils further analyses of mycorrhizal potential are recommended in order to continuously monitor the changes in ectomycorrhizal communities after the reductions of the emissions from the Thermal Power Plant Šoštanj.

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The morphological and genetical characterisation of a native Norway spruce (*Picea abies* (L.) Karst.) population in the area of Pokljuka mire

Morfološke in genetske značilnosti naravne populacije smreke *(Picea abies* (L.) Karst.) na območju pokljuškega barja

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Abstract. Morphometrical and genetical characteristics of two morphologically different sub-populations of spruce trees grown in a frost area of the mire Šijec (altitude 1170 m) on the Pokljuka plateau were studied. The tree height & diameter, needle length & needle volume of 70, approximately 120 to 200 years old trees, were measured. The genetic structure of the two spruce sub-populations were estimated by isozyme analysis on starch gel at 17 polymorphic gene loci. Soil conditions on the research plots examined with a gauge sound and then mapped and investigated in details by lab analyses of soil samples from representative soil profiles. The analysed spruce trees grown on the more productive site belonging to the association *Rhytidiadelpho lorei - Piceetum* in edge of the mire with mainly automorphic soils were in average 3,8 times taller and thicker than the trees from the extreme site belonging to the association *Sphagno girgensohnii - Piceetum.* The needle length of these trees was 25,7 % longer and their needle volume 68 % bigger than of the trees from the mire plot on the hydromorphic peat soils. The comparison of the genetic structures has shown distinct differences in frequencies of some alleles and genotypes between the analysed spruce sub-populations.

Key words: *Picea abies* (L.) Karst., sub-population, soil conditions, morphological variability, genetic structure, Julian Alps, Slovenia

Izvleček. Na Pokljuški planoti smo na mraziščnem področju barja Šijec (na n. v. 1170 m) proučevali morfološke in genetske značilnosti dveh različnih smrekovih subpopulacij. Okoli 120 do 200 let starim smrekam smo izmerili drevesne višine, premere debel v prsni višini ter dolžine in volumne iglic. Genetsko strukturo obeh subpopulacij smo proučili z elektroforetsko analizo 17 polimorfnih izoencimskih genskih lokusov v škrobnem gelu. Analizirane smreke z roba barja, ki rastejo na rastišču asociacije *Rhytidiadelpho lorei - Piceetum*, na katerem prevladujejo trdinska tla in je rodovitnejše, so imele v povprečju 3,8 krat večjo drevesno višino in premer ter za 25,7 % daljše iglice in za 68 % večjo prostornino iglic od smrek barjanske ploskve, ki poraščajo ekstremno rastišče asociacije *Sphagno girgensohnii - Piceetum*

na hidromorfnih šotnih tleh. Primerjava genetskih struktur je pokazala izrazite razlike v frekvencah nakaterih alelov in genotipov med analiziranima smrekovima subpopuacijama.

Ključne besede: *Picea abies* (L.) Karst., subpopulacija, talne razmere, morfološka variabilnost, genetska struktura, Julijske Alpe, Slovenija

Introduction

Forests cover about 20 km^2 of the Pokljuka plateau is calcareous and mixed moraines. Natural forests of beech (*Fagus sylvatica* L.) and fir (*Abies alba* Mil.) and secondary forests of Norway spruce (*Picea abies* (L.) Karst.) predominate, both with a high wood productivity. At the altitude of about 1200 m a.s.l. primary and supposedly autochthonous Norway spruce forests are still present and form two plant associations.

The spruce plant association *Rhytidiadelpho lorei - Piceetum* (ZUPANČIČ 1981 emend.) covers automorphic soils developed on moraines, spruce growth of these stands is relatively good. The spruce association *Sphagno girgensohnii - Piceetum* (W. KUOCH 1954 corr. ZUPANČIČ 1982) var. geogr. *Carex brizoides* (ZUPANČIČ 1982 corr.) grows on hydromorphic soils of mires. This spruce-mire plant community on peat soils lives in extremely poor and severe site conditions, spruces there grow slowly and dwarfly.

The aim of this study was to evaluate the influence of different site conditions on spruce growth characteristics and to determine the genetic structure of this species in two primary Norway spruce associations.

Materials and methods

Two research plots were established in the natural growing sites of spruce in the frosty area on the Pokljuka plateau. The research plot "Mire" lies in the south-eastern part of the mire Šijec in the bushy and gappy spruce forest (association *Sphagno girgensohnii-Piceetum*) between dwarf-pine community (*Sphagno-Pinetum mughi*) and high spruce forest on the mire's edge. The research plot "Edge" is located inside a one-hectare large permanent plot "Šijec" of the Slovenian Forestry Institute where sites of the spruce association *Rhytidiadelpho lorei-Piceetum* predominate (Figure 1). The plots are located at 1170 m altitude, the distance between them is 500 m.

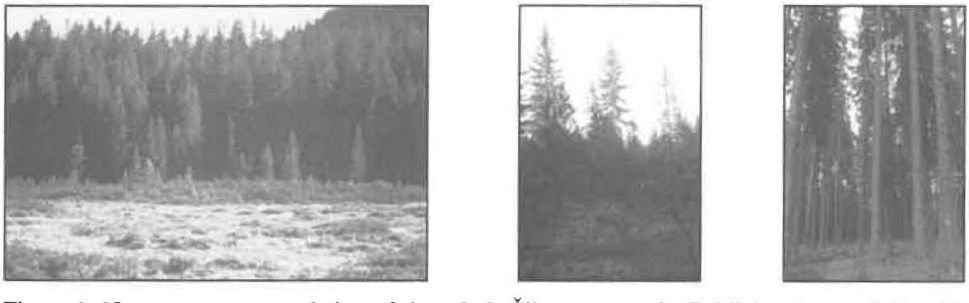

Figure 1: Norway spruce population of the mire's Šijec area on the Pokljuka plateau (left) with locations of the research plots "Mire" (in the middle) and "Edge" (right). Photo: G. Božič

Trees from every plot are considered as a sub-population from the whole spruce population of the mire's Šijec area. On each plot soil conditions were studied and 35 vital randomly chosen dominant spruce trees were dendrometrically analysed and their genetic structure determined.

Soil conditions and morphologic properties of soil in the research plots (see also BOŽIČ & URBANČIČ 2001) were examined with a semicircular sound, which reaches down to 110 cm deep. According to soil heterogeneity of plots the locations for representative soil profiles were chosen. After the description of profiles soil samples were taken and analysed in the lab. For each soil sample pH in de-ionised water (H_2O) and in 0.01 M calcium chloride ($CaCl_2$), content of: carbonates, organic carbon, organic matter, total nitrogen and C/N ratio were determined. Contents of exchangeable cations (K^+, Ca^{2+}, Mg^{2+}, Al^{3+}, Fe^{3+}, Mn^{2+} and H^+), cation exchange capacities, base saturation and texture classes were determined for samples from mineral part of soil.

The morphological characteristics of the sub-populations were determined by analysis of morphometric measurements of needle lengths and volumes and by dendrometric analyses of heights and diameters at the height of 1,3 m of dominant, approximately 120 to 200 years old spruce trees. Studied samples were taken from the 2 years old shoots exposed to sun in the southern upper third of the tree crown of 70 randomly chosen trees. Each tree sample consisted of 100 randomly chosen needles. All samples were taken during the second week in November and stored in the laboratory at –20°C until further use. Morphometric analysis of fully developed *Picea abies* needles were done by using a computer – aided image system (Optimas 5.0 programme) in the laboratory of Applied Botany and Physiology, Agronomy Department of Biotechnical Faculty, University of Ljubljana, under the supervision of MSc. T. Sinkovič. For calculation of needle volume (V) the equation of RIEDERER *et. al.* (1988) was used: $V\ (mm^3) = 0{,}208 \times (\text{projected needle area})^{1{,}353}$. The age of trees was estimated by measurments of tree rings from the cores taken at 0,4 m using a dendrocronological table LINTAB with 1/100 mm accuracy (BOŽIČ & LEVANIČ 1998).

To determine the genetic structure of the sampled trees, endosperms from six seeds per tree were analysed. In the mire's spruce sub-population there was no fructification during analysis, so dormant winter buds were used for analysis. The genetic variability of the two spruce sub-populations was analysed by means of isozyme gene markers using starch electrophoresis as the separation method. Enzyme extraction from buds and seeds (endosperm) was performed according to RHODES (1977). Electrophoresis conditions, staining, and genotyping followed KONNERT & MAURER (1995). The following isozyme gene loci were investigated: *Aco-A*, *Gdh-A*, *Got-A, Got-B, Got-C*, *Idh-A, Idh-B, Lap-B*, *Mdh-A, Mdh-B, Mdh-C*, *Mnr-A, Mnr-C*, *Pgi-A, Pgi-B*, *Pgm-A*, *Skdh-A*, *6-Pgdh-A, 6-Pgdh-B, 6-Pgdh-C*. The results of isozyme electrophoretic analyses were evaluated by relative allele and genotype frequencies calculated on different gene loci (recalculated from BOŽIČ 1997). The genetic structure was described for all loci for which polymorphisms were found in at least one spruce sub-population and compared with respect to the average actual heterozygosity H_a calculated as the arithmetic mean of single locus values (NEI 1973). The genetic differentiation between the sub-populations was studied with chi-square tests of homogeneity among allele frequencies for particular gene loci at the level $\alpha = 0{,}05$ and estimated with allelic and genotipic genetic distances (d_0) proposed by GREGORIUS (1974).

Results and discussion

On the bog site plot "Mire" all 35 studied spruce trees grow on hydromorphic organic soils which have the peat horizon lying over wet, softy and gelatinous lake sediments (gyttia). Thickness of the peat layer is from about 60 cm to over one meter. The reaction of its peat is very acidic

(measured values of pH($CaCl_2$) are between 2,88 and 3,18). Soil is classified as ombrotrophic form of the middle deep to deep subtype of peat acrohistosol and according to FAO (1989) soil classification it belongs to soil unit of Fibric Histosols. On this site spruces have extremely bad growth conditions. The ages (after BOŽIČ & LEVANIČ 1998) of spruces on the plot "Mire" at the height of 0,4 m varied between 65 and 142 (in average 95) years, at breast height they had diameters from 6 to 19 (in average 12) centimetres and tree heights from 4 to 13 (in average 8) metres. Analysed needles had lengths from 7,2 to 14,3 (in average 10,5) milimeters and volumes from 0,8 to 3,9 (in average 1,9) mm^3.

On the plot "Edge" soils have developed on mixed moraine lying over lake chalk. Mixed moraine is composed by unconsolidated material of limestone, dolomite, marl, cherts, shales and sandstones. On this parent material heterogenous, mostly dystric soils have developed. They are covered with mainly acidophilic vegetation. Soil sounding discovered that 14 sampled trees grow on dystric cambisols, 16 on podzols and 5 on gleysols. Properties of these FAO (1989) soil units were examined closely by three representative soil profiles. Podzols and dystric cambisols have in upper half meter depth very acid reaction (pH 3,02–4,37) and very low base saturation levels (BS 3,15 %–34,17 %). Gleysols are under the influence of groundwater and lake ckalk and are less dystric (pH 3,57–4,9; BS 43,9 %–53,8 %). These soils are rather deep, have suitable loamy texture and moisture regime and are rather fertile for spruce. The age of studied spruces at the height of 0,4 m in the plot "Edge" varied between 87 and 147 (in average 116) years, they had diameters at breast height from 31 to 60 (in average 46) centimetres and tree heights from 27 to 36 (in average 31) metres. Analysed needles had lengths from 9,9 to 17,5 (in average 13,2) milimeters and volumes from 1,0 to 6,3 (in average 3,2) mm^3 (modified after BOŽIČ 2000 and BOŽIČ & LEVANIČ 1998).

Also according to (BOŽIČ & LEVANIČ 1998) true ages of selected trees on the plot "Mire" were in average about 40 to 50 years greater and on the plot "Edge" from 30 to 35 years greater as in the sampling height 0,4 m. The age of the oldest trees is about 200 years, which means that trees in the two research plots started to grow at least 50 years before the first huge clearcuts and artificial regeneration with planting was started on the Pokljuka plateau in the years 1848–1859 (SMOLEJ 1984).

Between two sampled groups of trees on the research plots "Mire" and "Edge" remarkable differences in allele and genotype frequencies were noted. The most distinct differences in frequency of alleles and genotypes was observed at locus *Mnr-A* for alleles *Mnr-*A_2, *Mnr-*A_4, and genotypes *Mnr-*A_{22} and *Mnr-*A_{24}. The homozygote type was more common in sampled trees from the plot "Edge" while the hetrozygote type was more common in sampled trees from the plot "Mire". The heterozygote types *Mnr-*A_{24} *and Mnr-*A_{34} reached much higher values in sampled group of trees on the mire site than on the edge site. The reverse situation was occured at homozygote type *Mnr-*A_{22} which was much more frequent in group of trees "Edge" than in "Mire" group of trees (45,7 % vs. 11,4 %, respectively). In average, a higher heterozygosity (H_a) on the plot »Mire« then on the plot »Edge« was observed. If the most differentiated locus in heterozygosity *Mnr-A* is taken in consideration, trees sampled on the »Mire« had much higher heterozygosity as sampled group of trees on the »Edge« plot (77,1 % and 40,0 %, respectively). Results of the contingency χ^2 tests for homogeneity of genetic structure between sub-populations has shown that allele frequencies significantly differed at the level $\alpha = 0,05$ in two gene loci (chi-square = 8,13; 3 d.f. for *Lap-B*) and (chi-square = 5,27; 1 d.f. for *Mnr-A*). At this two gene loci, also the genetic distances between »Edge« and »Mire« group of trees were very high with allelic distances of 17,2 % for *Lap-B* and 21 % for *Mnr-A* and genotypic distances of 25,8 % for *Lap-B* and 37,2 % for *Mnr-A*. Remarkable differences of allele and genotype frequencies were also noted for loci *Aco-A*, *Got-C Lap-B, Mnr-A*

and *6-Pgdh-C*. Only for gene locus *Gdh-A*, the genetic structures of the two sub-populations were identical and the genetic distances equal with zero. The single-locus values mean value of Gregorius allelic and genotypic genetic distance (5,9 % and 10,7 %, respectively), further confirms that the genetic differences between the sampled spruce sub-populations on the "Mire" and "Edge" plots are comparatively high. For the population genetic structure of Norway spruce a relative high levels of differentiation between the sub-populations were observed.

Genetic differentiation between the sub-populations from true different forest site conditions may be a consequence of different selection processes to which spruce in this location has been exposed. As spruces from the mire site with organic (peat) soils were 3,8 times lower and thinner as spruces in similar age from the site with mineral soils, is there a great opportunity for selection to act. Since temperature and oxygen availability in the soil are closely associated with soil moisture, further experiments are required to understand the environmental factors which are most directly associated with the selection factors which acting on the forest sites with mainly automorphic (out of undergroud water reach) and mineral or hydromorphic (strong influence of undergroud water) peat soils.

The very high allelic and genotypic distances observed in loci *Lap-B* and *Mnr-A* indicated that specific site conditions could have an impact to these two loci. The response to site conditions could affect the observed locus directly, or any other loci tightly linked to them, as well as any coadapted gene complexes that they mark (HAMRICK 1989). As the isoenzyme variants (allozymes) may be both, selectively equivalent (i.e. neutral) and adaptive, depending on the respective environmental conditions under which the particular population is living (BERGMANN 1991), present results would allow to hypothesise that decrease in frequency of allele $MNR\text{-}A_2$ from 65,7 % observed in forest site with mainly automorphic and mineral soils to 44,3 % on mire site, may have an adaptive origin and could be connected with increased level of heterozigosity of individuals existing in environmental site conditions with hydromorphic soils because only heterotzigotic individuals can be better adapted to exteme site conditions.

Althought the obtained results are incomplete, also because of possible sampling mistakes on account of the large size of analysed samples, and do not allow us to infer any certain conclusions on the possible adaptive role of the enzyme systems under analysis, they verify the existence of morphological and genetical differentiation between spruce sub-populations within single population associated with variation of environmental conditions resulting from differences in site conditions.

Conclusions

The research plots were established on Pokljuka in sites within the area of natural distribution of Norway spruce. On both plots dominant from about 120 to 200 years old spruces were chosen. The oldest trees were growing at least 50 years before the first huge clearcuts and artificial regeneration with spruce seedlings on Pokljuka plateau in the middle of 19th century began so the analysed spruce sub-populations in the Šijec area can be regarded as autochthonous.

Striking differences in soil and other site conditions between the research plots have been reflected in the spruce's growth. Spruces from the plot "Edge" were in average 3,8 times taller and thicker as trees from the plot "Mire". Needle length and needle volume of spruces from the site with better growing conditions were 26 % longer and 68 % bigger than of the trees from the peat bog. Different site conditions resulting from soil conditions were attributed particularly to the tree growth and its morphological attributes.

Environmental conditions resulting from differences in site conditions could have an impact to the genetic structures of sub-populations growing in such habitats. The obtained results would also allow to hypothesise an adaptive response of the allele *Mnr-A*$_2$ to different environmental conditions resulting from differences in site conditions.

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Povzetek

Raziskovalni ploskvi sta bili osnovani v mraziščnem delu Pokljuke, v okolici visokega barja Šijec, v dveh smrekovih subpopulacijah, na njenih naravnih rastiščih. Na vsaki ploskvi smo za raziskavo izbrali 35 vitalnih, nadraslih oziroma soraslih smrek različnih starosti (od 120 do 200 let). Najstarejša drevesa so rastla vsaj 50 let pred prvimi velikopovršinskimi goloseki in sadnjami smrekovih sadik sredi 19 stoletja, zato menimo, da so analizirane smreke še avtohtone.

Ploskev »Mire«, ki jo porašča asociacija barjanskega smrekovja *(Sphagno - Piceetum* s. lat. R. KUOCH 1954 emend. ZUPANČIČ 1981), leži na šotnem barju. Njena srednje globoka do globoka šotna tla po FAO (1989) razvrstitvi spadajo v talno enoto fibrični histosoli. Na njej vladajo za smreko skrajno neugodne rastiščne razmere.

Ploskev »Edge« smo osnovali na robu barja. Njeno rastišče porašča rastlinska združba smreke in smrečnega resnika *(Rhytidiadelpho lorei - Piceetum* (M. WRABER 1953 n.nud.; ZUPANČIČ (1976), 1981 em. 1999). Na njem so se na mešani moreni, ki prekriva jezersko kredo, razvili podzoli, distrična rjava tla in hipogleji. Po mednarodni FAO (1989) klasifikaciji jih uvrščamo v talne enote: podzoli, distrični kambisoli in glejsoli. Ta tla so distrična (imajo zelo kisle reakcije in zelo nizke stopnje nasičenosti z izmenljivimi bazami) in so vsaj pol metra globoka. Imajo ugodno ilovnato teksturo in vodni režim, tako da so za smreko dokaj rodovitna.

Analizirane smreke z roba barja so bile v povprečju 3,8- krat višje in debelejše ter so imele v povprečju za 25,7 % daljše in za 68,4 % volumensko večje iglice od smrek s šotnega barja. Te razlike v rasti in v morfoloških lastnostih smrek so po našem prepričanju odraz velikih razlik v talnih in drugih rastiščnih razmerah med ploskvama.

Te razlike v okoljskih razmerah pa bi lahko vplivale tudi na genetski strukturi analiziranih smrekovih subpopuacij. Izidi naših analiz genetske variabilnosti subpopulacij smreke z metodo izoencimske gelske elektroforeze, ki je zajemala uporabo škrobnega gela in ekstrakcijo izoencimov iz popkov in endosperma semen, podpirajo to domnevo. Razlika v heterozigotnosti med subpopulacijama na genskem lokusu *Mnr-A* nakazuje možni učinek preživetvene selekcije.

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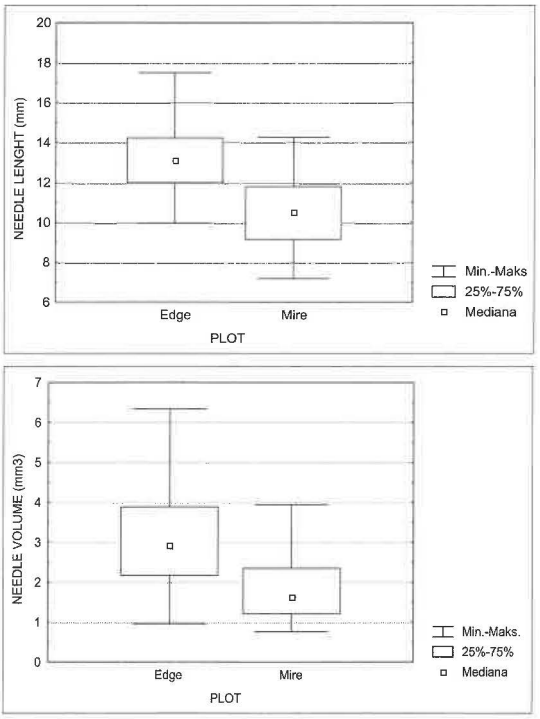
Appendices

Appendix 1: The tree age on the height of 0,4 m, diameter at 1,3 m, tree height, needle length and needle volume of sampled spruce trees on the research plots "Mire" and "Edge"

Parameter		Plot "Mire"	Plot "Edge"
Age	Min. (years)	65	87
	Max. (years)	142	147
	Mean (years)	95	116
Diameter	Min. (cm)	6	31
	Max. (cm)	19	60
	Mean (cm)	12	46
Height	Min. (m)	4	27
	Max. (m)	13	36
	Mean (m)	8	31
Needle length	Min. (mm)	7,2	9,9
	Max. (mm)	14,3	17,5
	Mean (mm)	10,5	13,2
Needle volume	Min. (mm^3)	0,8	1,0
	Max. (mm^3)	3,9	6,3
	Mean (mm^3)	1,9	3,2

Appendix 2: The distribution of needle length and needle volume values of sampled spruce needles per each research plot



Appendix 3: Genotype frequencies at the 20 analysed enzyme gene loci of sampled spruce trees on the research plots "Mire" and "Edge"

Locus	Genotype	Plot "Mire"	Plot "Edge"	Locus	Genotype	Plot "Mire"	Plot "Edge"
ACO - A	11	0,086	0,086	MDH - C	24	0,086	0,000
	12	0,200	0,343		44	0,914	1,000
	22	0,714	0,571				
				MNR - A	12	0,057	0,000
GDH - A	12	0,029	0,029		22	0,114	0,457
	22	0,971	0,971		24	0,600	0,400
					34	0,114	0,000
GOT - A	12	0,000	0,029		44	0,114	0,143
	22	1,000	0,971				
				MNR - C	12	0,057	0,000
GOT - B	12	0,000	0,029		22	0,943	1,000
	22	1,000	0,971				
				PGI - A	22	1,000	1,000
GOT - C	22	0,171	0,086				
	24	0,514	0,429	PGI - B	22	0,000	0,086
	25	0,029	0,029		23	0,600	0,514
	44	0,257	0,429		33	0,400	0,400
	45	0,029	0,029				
				PGM - A	22	0,829	0,857
IDH - A	23	0,029	0,114		23	0,143	0,114
	33	0,971	0,886		33	0,029	0,029
IDH - B	22	1,000	1,000	SKDH -A	13	0,029	0,057
					22	0,143	0,086
LAP - B	11	0,000	0,029		33	0,829	0,800
	13	0,000	0,029		35	0,000	0,029
	14	0,029	0,086		36	0,029	0,029
	33	0,000	0,029				
	34	0,114	0,200	6PGDH - A	12	0,000	0,029
	36	0,000	0,029		22	0,971	0,971
	44	0,571	0,429		23	0,029	0,000
	46	0,229	0,143				
	47	0,029	0,000	6PGDH - B	22	0,400	0,486
	66	0,029	0,029		25	0,486	0,343
					35	0,000	0,029
MDH - A	22	1,000	1,000		55	0,114	0,143
MDH - B	12	0,029	0,000	6PGDH - C	15	0,000	0,029
	22	0,914	0,971		22	0,457	0,314
	23	0,057	0,029		25	0,257	0,400
					26	0,029	0,000
					55	0,257	0,257

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Impact of air pollution on the mitotic activity in meristematic cells in shallot (*Allium cepa* L. var. *ascalonicum*)

Vpliv onesnaženega zraka na mitotsko aktivnost v meristemskih celicah šalotke (*Allium cepa* L. var. *ascalonicum*)

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Abstract. Test shallot plants *Allium cepa* L. var. *ascalonicum* were exposed to field conditions in research plots in the most polluted areas in Slovenia in the vegetation season in 1999. The intention of this research was to evaluate the influence of air pollution on mitotic activity in meristematic tissues of root tips of bioindication plants. At each sampling site the mitotic activity was determined under field conditions and in a pot experiment. The maturated bulbs were collected from the field and after winter dormancies cytogenetic analyses were run on them in a lab. Root tips were fixed in Clark´s fixative and afterwards stained with Schiff´s reagent.

Significant differences in the mitotic activity in different sampling plots in pot experiments were found. The results showed the presence of cytotoxic substances at chosen sampling sites which caused the decrease of mitotic cell division.

Key words: *Allium cepa* L. var. *ascalonicum*, cytotoxicity, mitotic index, environmental pollution.

Izvleček. Izbrane testne rastline šalotke (*Allium cepa* L. var. *ascalonicum*) so bile v času vegetacijske dobe izpostavljene naravnim razmeram na vzorčna mesta v bližini virov onesnaženja na ozemlju Slovenije. Namen raziskave je bil oceniti vpliv onesnaženega zraka na mitotsko aktivnost v meristemskih celicah koreninskih vršičkov bioindikatorskih rastlin. Vpliv onesnaženega okolja smo analizirali pri rastlinah izpostavljenih v lončnem in poljskem poskusu.

Posušene in očiščene čebulice so bile po zimski dormanci izpostavljene v laboratoriju, koreninski vršički so bili fiksirani v Clarkoven fiksativu in obarvani s Schiffovim reagentom.

Ugotovljeno je bilo, da se pojavljajo statistično značilne razlike med posameznimi lokacijami pri rastlinah izpostavljenih v lončnem poskusu. Rezultati kažejo, da so na izbranih lokacijah citotoksične substance, ki vplivajo na mitotsko aktivnost celic izpostavljenih rastlin.

Ključne besede: *Allium cepa* L. var. *ascalonicum*, citotoksičnost, mitotski indeks, onesnaženost okolja.

Introduction

Besides the analysis of chromosomal aberrations to evaluate genotoxicity a mitotic index is most frequently used as a parameter to evaluate cytotoxicity to show effect of chosen substances in cytogenetic bioindication. Mitotic activity decreases while the influence of environmental stress is stronger (Fiskesjö 1994, Paradiž 1996, 1998, Paradiž & al. 1996, Smaka-Kincl & al. 1996, Bavcon & al. 1999, Freiszmuth & al. 2000, Müller & al. 2000, Pavlica & al. 2000, Paradiž & Druškovič, 2001). In the *Alliaceae* family shallot is not commonly used for bioindication (Pavlica & al. 1997, 2000, Zoldoš & al. 1997).

Test shallot plants (*Allium cepa* L. var. *ascalonicum*) were exposed to field conditions at research plots of differently polluted areas of Slovenia in 1999: in the Šalek Valley the most polluted sites are either those which are very close to the thermal power plant (Veliki Vrh, ash dump of the Šoštanj Thermal Power Plant) or those which are highly polluted due to climatic conditions and topography but more distant from the thermal power plant (Zavodnje) or sites with relatively clean air in relation to the wind direction from the thermal power plant (Arnače, Škale); in the Zasavje region (Kovk) and in the Upper Meža Valley (Žerjav) two very polluted sites were taken for one vegetation period. At each sampling plot plants were growing under field conditions and pot experiment (see *Material and methods* for a short description of growing conditions). Mitotic activity was later determined in a laboratory.

In this study we attempted to determine the influence of air pollutants on plants (pot) and indirect, accumulated effects in soil exposed to environmental pollution on the cytogenetic material of the shallot. The aims of the study were to make a comparison among the sampling plots regarding the mitotic activity to define cytotoxic effect arising from environmental pollution and to confirm the applicability of chosen method for relatively fast, simple and cost-effective determination of cytotoxicity of polluted air. In comparison with the pots experiments (where plants were exposed primarily to air pollution) we expected decrease of mitotic activity in plants in field conditions, since these shallots were exposed to stress due to air and soil pollution.

Materials and methods

For every sampling site we used approximately equal-sized shallot bulbs (*Allium cepa* L. var *ascalonicum* Escalote de Jersey', 2n=16).

The choice of sampling sites and sampling procedure was carried out as described by Glasenčnik & al. (2002) and the method for cytogenetic analyses was performed as described by Fiskesjö (1994) and detailed by Glasenčnik & al. (2002).

In the pot experiment plants were exposed in 15-litre self-watering pots (five per site, three bulbs per pot) and for experiment under field conditions plants were put directly in field soil (six bulbs per site) near the pots in the end of May 1999. After maturation all plant material was removed from sampling plots and bulbs were stored under dry conditions (4°C) for two months.

The soil from pots and field soil were analysed. The analysis of soils was carried out after homogenisation (ISO 11464) and digestionation (ISO 11466) using the methods for spectroscopy (ICP-MS, ET-AAS, CV-AAS).

Cytogenetic analyses were run after winter dormancies. Root tips were treated with using Feulgen "squash" technic. A mitotic activity was determined in five bulbs per sampling site, one slide per bulb and 2000 cells per slide. A mitotic index (MI) is a percentage of dividing cells scored.

All statistical treatments were performed using the Statistica for Windows 5.5 software package (STATSOFT 1999). Differences among sampling sites were tested using Kruskal-Wallis ANOVA. Cross comparisons between means were performed using the Mann-Whitney U test.

Results

Significant differences were found in MI among the sampling sites for shallots grown in pots (Kruskal-Wallis ANOVA: $H_{(6, 47)} = 19{,}49$, $p = 0{,}003$; *Fig. 1b*), while for plants grown in field soils no significant differences could be found ($H_{(5, 41)} = 7{,}68$, $p = 0{,}17$; *Fig. 1a*). Detailed statistics of pair-wise comparisons using Mann-Whitney U test are presented in *Table 1*.

The analysed plants material in pot experiment showed that the MI was between 3 and 14 %. The lowest value of MI was determined in Arnače near Velenje (3,7±2,5 %), a very low value was also at Veliki Vrh and at Kovk (6,1±2,6 % and 6,1±1,6 %), the highest was at Žerjav (10,93±3,5 %).

The results of shallot grown under field conditions showed that the value of MI was between 6 and 10 %, the lowest was determined at Veliki Vrh (6,4±3,7 %), the highest at the sampling site of Žerjav (10,93±3,5 %).

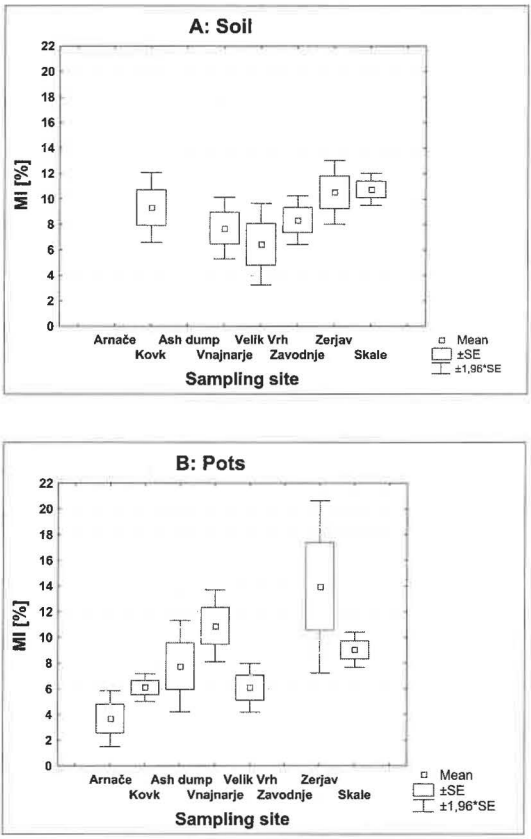

Figure 1a, b: Mitotic activity (%) in root tip cells of shallot, exposed to field conditions in soils (A) and in pots (B) at eight different localities in Slovenia in the vegetation season in 1999.

Table 1: Significance of differences in mitotic activity between sampling sites (Mann-Whitney U-test).

		Soil							
	1999 **MI**	ARNAČE	KOVK	ASH DUMP	ŠKALE	VNAJNARJE	VELIKI VRH	ZAVODNJE	ŽERJAV
Pots	ARNAČE		/	/	/	/	/	/	/
	KOVK	*		/	NS	NS	NS	NS	NS
	ASH DUMP	NS	NS		/	/	/	/	/
	ŠKALE	**	**	NS		NS	*	NS	NS
	VNAJNARJE	*	*	NS	NS		NS	/	NS
	VELIKI VRH	NS	NS	NS	*	*		NS	NS
	ZAVODNJE	/	/	/	/	/	/		NS
	ŽERJAV	*	/	NS	NS	NS	NS	*	

***: p<0.001; **: p<0.01, *: p<0.05; NS: not significant.

Discussion

The inhibition of mitotic activity was often used for tracing cytotoxic substances. Cytotoxicity was defined as a decrease in the mitotic index and as an increase in the frequencies of chromosomal aberrations (Fiskesjö 1994). The analysis of chromosomal aberrations for evaluation of genotoxicity was already done (for results see Glasenčnik & al. 2002). In our study a decrease in the mitotic index of shallot root meristems was found depending on the chosen location in pot experiment with probability p<0,01. We used the same soil for all pots and therefore plants grown in pots were exposed only to air pollutants. Analysis of chromosomal aberrations showed no significant differences, but the frequency of chromosomal aberrations was at least two-fold higher in comparison to published data for a control environment (3%, e.g. Vidakovič & al. 1993). Therefore air pollution had a strong influence on mitotic activity and also on the frequency of chromosomal aberrations. These results are in accordance with the results of fumigation experiments of a research group in Graz: ozone treatments consistently increased the rate of chromosomal aberrations, the MI remained unchanged, with fumigation with SO_2 and H_2S the MI was lower and the chromosomal aberrations were also consistently increasing (Müller & al. 2000).

Arnače sampling plot showed, against expectation, a very small mitotic activity, though the finding was in accordance with the recent results from other research fields (Svetina 1999, Kugonič & Stropnik 2001), which revealed elevated levels of Co, Cr and Ni in meadow soil and higher levels of heavy metals in vegetables and forage were found in comparison with other sampling sites in the bottom of the Šalek Valley. The highest frequency of chromosomal aberrations (for details see Glasenčnik & al. 2002) in our pot experiment confirmed the presence of stress inducing factor at this sampling site.

Since the cleaning device was built on the Šoštanj Power Plant in 1995, the emissions have been drastically reduced, which has reflected in an improvement of ecosystem conditions (Ribarič-Lasnik & al. 1999b). This is not a case at some sites, which are still polluted due to their position or due to accumulation of pollutants in the past. At the sampling site of Veliki Vrh just facing power plant chimneys and the prevailing wind direction a high concentration of air pollutants is directly brought from chimneys to this highly exposed site. This was confirmed by several studies (e.g. high levels of Cd in soil and vegetables Kugonič & Stropnik 2001, high levels of Cd, As and Hg in fungi Al Sayegh-Petkovšek & al. 2002, the most acid precipitation (pH<5,6) Sevšek & al. 2000). The low value of MI determined and the highest frequences of chromosomal aberrations (see Glasenčnik & al. 2002) at that sampling site were in agrement with the above mentioned studies.

Considering the extreme pollution with heavy metals in the Upper Meža Valley (see RIBARIČ-LASNIK & al. 1999a and references therein) the high value of MI in both experimented plots in Žerjav were surprising because we expected a low value of MI at this site due to extreme value of heavy metals in the surroundings (e.g. 4,99±6,92 $mgkg^{-1}$Cd, 1496±1071,02 $mgkg^{-1}$ Pb, 498,22±546,0 $mgkg^{-1}$ Zn KUGONIČ & STROPNIK 2001). More investigations are needed at this research plot to verify the results.

Contrary to our expectation no significant differences in the mitotic activity could be found among localities in the experiment under field conditions. We found significant differences in the frequency of chromosomal aberrations in different sampling sites concerning shallot grown in the field (see GLASENČNIK & al. 2002). The results are in accordance with the statement of FISKESJÖ (1997) saying that when chromosomal aberrations occur, there are almost always some growth restrictions. These results indicated that the influenece of chronic exposure (soil) is far more genotoxic while the acute exposure (pots) is more cytotoxic. Results obtained with our investigation were confirmed also with the results of an assessment of cytogenetic hazard for plants caused by highway traffic: the mitotic activity decrease and the frequency of chromosomal aberrations increase with the duration of traffic influence in the onion (*Allium cepa* L.) exposed in short-lasting *in situ* exposure near highways in Slovenia (PARADIŽ & DRUŠKOVIČ 2001). In spruce and other perennial plants growing spontaneously on experimental sites near the highways as bioindicators of long-lasting exposure, cytogenetic damages increased in correlation with the duration of traffic, but only at sites with more than 20 years' traffic influence (*ibid*).

Nevertheless, we also found out that the values of MI in the field conditions and in the pot experiment were lower than the average values from the literature for the control (10-15 %; e. g. Oud & RICKARDS 1999; 5-10%; e. g. PARADIŽ 1996) and these results showed a decrease in mitotic activity and a cytotoxic effect of chosen sites.

Considering the present results we can conclude that the polluted air does have a cytotoxic effect on mitotic activity with the decrease of MI and that more polluted sites have a higher effect on mitotic activity. We can also say that the polluted air and soil together probably had a stronger effect at the majority of investigated plots. Therefore we conclude that the mitotic activity is the appropriate testing system for determination of MI in shallot root tip meristematic cells for quick, easy and useful estimation of cytotoxic effect of environmental pollution.

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Growth and root respiration of C4 plants under CO_2 enrichment

Rast in dihanje korenin C4 rastlin pri povečani koncentraciji CO_2

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Abstract. Respiratory measurements of apical root parts of several C4 plant species (*Echinochloa crus-galli* var. *crus-galli*, *Setaria pumila* and *Zea mays* DK 312 (Dekalb, USA) subjected to an elevated CO_2 regime during growth in climatic chambers or at natural CO_2 springs were performed.

Biomass production, root respiratory potential and root respiration of *Echinochloa* was not significantly changed by high atmospheric CO_2 treatment in the climatic chambers, compared to ambient CO_2 treatment.

Root respiratory potential of C4 weeds (*Echinochloa crus-galli* and *Setaria pumila*) growing in natural CO_2 spring area was not significantly affected by extremely high CO_2 in the rhizosphere. Yet, respiratory potential of one and a half month old sown maize seedlings was significantly lower in the roots exposed to naturally elevated CO_2 concentrations.

Key words: root respiration, ETS activity, respiratory potential, C4 plants, *Echinochloa crus-galli*, *Zea mays*, *Setaria pumila*, elevated CO_2, natural CO_2 springs, CO_2 mofette

Izvleček. V pričujoči raziskavi smo merili dihalno aktivnost apikalnih delov korenin nekaterih C4 vrst (navadne kostrebe *Echinochloa crus-galli* var. *crus-galli*, sivozelenega muhviča *Setaria pumila* in koruze *Zea mays* DK 312 (Dekalb, ZDA), izpostavljenih povečani koncentraciji CO_2 med rastjo v klimatskih komorah ali ob naravnih izvirih CO_2.

Pri navadni kostrebi povečana koncentracija CO_2 v klimatskih komorah ni značilno vplivala na produkcijo biomase, dihalni potencial in dihanje korenin.

Ekstremno povečana koncentracija CO_2 v rizosferi rastlin (navadne kostrebe in sivozelenega muhviča) ob naravnih izvirih CO_2 ni značilno vplivala na dihalni potencial v koreninah. Ta je bil značilno manjši le pri mesec in pol stari sejani koruzi rastoči na področju naravnih izvirov CO_2.

Ključne besede: dihanje korenin, aktivnost ETS, dihalni potencial, C4 rastline, *Echinochloa crus-galli*, *Zea mays*, *Setaria pumila*, povečana koncentracija CO_2, naravni izviri CO_2, CO_2 mofeta

Introduction

An elevated atmospheric CO_2 concentration can have a significant effect on growth and carbon metabolism of many plant species. On a daily basis, more than 50% of the photosynthates produced, may be simultaneously respired by roots (LAMBERS et al. 2002). Compared to the number of papers on inhibition of aboveground shoot respiration by elevated CO_2 (e.g. AMTHOR 1991, DRAKE et al. 1997, TJOELKER et al. 2001), very little information on the effects of elevated CO_2 on root respiration has been published so far. Furthermore, the reported CO_2 effects on root respiration are rather heterogeneous, limited to only few plant species and differently discussed among authors (e.g. BURTON et al. 1997, YODER et al. 2000, LAMBERS et al. 2002). LAMBERS et. al. (1996) report that there is insufficient evidence to state that root respiration is inhibited by the [CO_2] around roots in a similar manner as leaf respiration is inhibited by elevated CO_2 in the atmosphere. Moreover, there is no convincing evidence for a direct effect of elevated atmospheric CO_2 on the specific rate of root respiration or the fraction of carbon required for root respiration. However, there are probable indirect effects of elevated CO_2 on the carbon requirement of plants in natural systems.

The partial pressure of CO_2 in soil may differ from the CO_2 partial pressure above ground and it seems to be more variable. The concentration of CO_2 in soil rapidly increases after rainfall because its diffusion through the gas phase is restricted by the water saturation of the soil. Thus, plant roots are frequently exposed to relatively high CO_2 concentrations. Water flooding can also present a longer exposure of roots to elevated CO_2 and consequently hypoxic environment. Plants growing near CO_2 springs are exposed to extreme CO_2 regimes that can also lead to hypoxic conditions in the rhizosphere. Vegetation at CO_2 springs has been exposed to such conditions for long periods, giving time for acclimation, and perhaps also genetic adaptation of plants (VODNIK et al. 2002). Thus, natural CO_2 springs may represent a good model ecosystem to study effects of elevated CO_2 on plants (RASCHI et al. 1997, BADIANI et al. 1999).

Our work was performed on three C4 plant species *Echinochloa crus-galli* var. *crus-galli*, *Setaria pumila* and *Zea mays* DK 312 (Dekalb, USA) subjected to an elevated CO_2 regime during growth in climatic chambers as well as in a CO_2 spring-area Stavešinci in NE Slovenia.

Material and methods

Seedlings of *Echinochloa*, the offsprings of plants growing near the CO_2 springs, were grown in climatic chambers under ambient (368.4 ± 15.6 μmol CO_2 mol^{-1}) and elevated (1906.2 ± 195.0 μmol CO_2 mol^{-1}) CO_2 for seven weeks at the temperature 25 °C during the light (14 h) and 16 °C during the dark period (10 h) of the day. The fumigation started two weeks after seed germination. After the growth period plant roots were sampled for respiratory measurements.

In the July 2002, root samples were also collected for natural growing *Echinochloa*, *Setaria* and one and a half month old plants of sown maize (*Zea mays* DK 312, Dekalb, USA) growing in the natural CO_2 spring area Stavešinci in NE Slovenia (for detailed information on the site characteristics and other details see VODNIK et al. in press). The plants were chosen according to their height and the preliminary measured soil CO_2 concentration in the rooting zone by a gas analyzer GA 2000 (Ansyco, FRG). A significant correlation between plant height and plant exposition to elevated CO_2 from CO_2 springs is known from our previous studies (VODNIK et al. 2002[a,b], TURK et al. 2002).

Root respiration rates were measured as oxygen consumption on root tip segments (1 cm length) using Clark-type oxygen electrodes (Hansatech, Norfolk, UK). Measurements were performed in 50 mM MES (morpholinoethanesulfonic acid) buffer solution pH 6 at 20 °C.

The root respiratory potential - electron transport system (ETS) activity was determined on root tip segments (1 cm length) using the iodonitrotetrazolium salt (INT) method described by KENNER & AHMED (1975).

Statistical analyses were performed by Statgraphic Plus 4.0 (Statistical Graphics Corp.).

Results and discussion

In the fumigation experiment no significant impact of high atmospheric CO_2 concentration on biomass production of *Echinochloa* was found (Tab. 1). In general, the growth response of C4 plants to elevated [CO_2] is inconsistent and depends on various environmental factors (GHANNOUM et al. 2000). For *Echinochloa* the increase of plant biomass under elevated CO_2 concentration (690 µmol mol^{-1}) was documented by ZISKA et al. (1997).

Measurements of root oxygen consumption revealed no significant differences in root respiration of elevated CO_2 chamber-grown *Echinochloa* plants. There was also no significant difference in root respiratory potential (Tab. 1).

Table 1: Biomass production, root respiration and root respiratory potential of *E. crus-galli* var. *crus-galli* subjected to elevated atmospheric CO_2 in climatic chambers, (avg ±SD).

	Ambient CO_2 (368.4 ± 15.6 µmol CO_2 mol^{-1})	Elevated CO_2 (1906.2 ± 195.0 µmol CO_2 mol^{-1})
Dry weight of shoot (g) [(1)]	0.95 ± 0.26	1.15 ± 0.26
Dry weight of root (g) [(1)]	0.83 ± 0.16	0.89 ± 0.33
Root respiration[(2)]	1.5 ± 0.6	1.8 ± 0.5
ETS roots[(3)]	1.0 ± 0.2	1.0 ± 0.2

[(1)]By ANOVA, n = 24. [(2)]Given as nmol O_2 g^{-1} fresh wt s^{-1}, by ANOVA, n = 15. [(3)]Given as µg O_2 g^{-1} fresh wt h^{-1}, by ANOVA, n = 15

In addition to these findings, measurements of *Echinochloa* exposed to different CO_2 regimes at the natural CO_2 spring Stavešinci showed no significant effects of high rhizospheric CO_2 concentration on root respiratory potential of the root-tip segments. The same was true for another C4 plant *Setaria pumila* (Tab. 2). It is to conclude that both species are relatively insensitive to high CO_2. A high tolerance of *E. crus-galli* to hypoxia is known from different studies and is especially well documented for its variety *E. crus-galli* var. *oryzicola* (BUCHANAN & al. 2000). Germination of *E. crus-galli* could be stimulated by elevated CO_2 as it was shown by YOSHIOKA & al. (1998). In this study germination was stimulated by exposure to 30 mmol mol^{-1} CO_2 and it was concluded that soil CO_2 is responsible for causing intermittent flushes of seed germination of this species after heavy rainfall. This could also explain the presence of germinating and growing *Echinochloa* plants at the sites

with extreme CO_2 concentrations in the natural CO_2 spring Stavešinci as reported by KALIGARIČ (2001). No similar reports have been published on *Setaria pumila*.

Table 2: Shoot height and root respiratory potential of *E. crus-galli* var. *crus-galli*, *S. pumila* and *Z. mays* subjected to elevated soil and atmospheric CO_2 at a natural CO_2 spring (avg ± SD).

Plant species	CO_2 exposure[1]	Mean height (cm)[2]	ETS[3]
Echinochloa crus-galli	Low (0.4%)	62.3 ± 11.7	1.52 ± 0.19
	High (26%)	15.9 ± 1.8	1.55 ± 0.18
Setaria pumila	Low (0.4%)	51.0 ± 6.8	0.34 ± 0.05
	High (26%)	28.0 ± 5.6	0.32 ± 0.06
Zea mays	Low (0.1–0.4%)	239.0 ± 21.0	1.12 ± 0.13
	High (over 10%)	114.2 ± 7.9	0.95 ± 0.13

[1]Measured as soil CO_2 concentration (25 cm depth) by a gas analyzer GA 2000 (Ansyco, FRG). [2]By ANOVA, n = 10. [3]Given as μg O_2 g^{-1} fresh wt h^{-1}, by ANOVA, n = 12.

Results on *Echinochloa* and *Setaria*, could indicate a general low sensitivity of root respiration to a high CO_2 concentration, which is suggested by LAMBERS et al. (1996, 2002). Yet, root respiratory potential in root tips of *Zea mays* measured in our study was significantly lower in the roots exposed to high soil CO_2 than in those growing in the low CO_2 environment (Tab. 2). Different results obtained for native species (*Echinochloa*, *Setaria*) and sown maize suggest that plants growing as a part of natural vegetation could be adapted to extreme conditions. This however, has to be confirmed in the future work.

Conclusions

At natural CO_2 springs, the growth of *Echinochloa* and *Setaria* can be decreased by high CO_2 concentrations and physiological processes in shoots can be severely affected. Despite this, no significant impact of CO_2 exposure on root respiratory potential of the root-tip segments was found for the same species. More detailed physiological studies on root respiration are needed, regarding to the different parts of the root system, different ontogenetic development and measurements on different plant species. Further research is also needed in connection to different environmental factors affecting root respiration.

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Influence of *in vitro* propagation on the economically important traits of strawberry cv. Marmolada

Vpliv *in vitro* razmnoževanja na ekonomsko pomembne lastnosti jagod cv. Marmolada

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Abstract. *In vitro* propagation of strawberries is a promising alternative to traditional propagation, since it provides better sanitary status of plants. The possibility of somaclonal variation presents one of the drawbacks of this method, but this phenomena can be minimised by the choice of optimal tissue culture procedure.

To evaluate the influence of *in vitro* growing on economically important traits of strawberry cv. Marmolada, rooted plants were produced *in vitro* from a long term and from a newly established culture. These plants were used as mother plants for short multiplication in the field. Vegetative and generative traits of their runner plants were compared with conventionally produced cold storage plants and plants derived directly from tissue culture. Statistically significant differences were observed among different plant types in the number of runners per plant, number of flowers per plant, number of fruits per plant and yield per plant. The most pronounced differences were observed in the number of fruits per plant and in the yield per plant. Plants obtained directly from tissue culture were almost twice as productive as conventionally produced cold storage plants. Conventionally produced cold storage plants had statistically significantly higher yields and fruit number per plant than runner plants derived from micropropagated mother plants. High yields of plants obtained directly from *in vitro* were mainly the results of their significantly prolonged ripening.

Keywords: strawberry, micropropagation, yield, quality, somaclonal variation

Izvleček. *In vitro* razmnoževanje jagod predstavlja zanimivo alternativo klasičnemu razmnoževanju, saj zagotavlja boljše zdravstveno stanje sadik. Somaklonska variabilnost je lahko omejujoč dejavnik pri uveljavljanju mikrorazmnoževanja jagod, vendar lahko z izbiro optimalne *in vitro* tehnike ta pojav znižamo na minimum.

Za ugotavljanje vpliva *in vitro* gojenja na ekonomsko pomembne lastnosti jagod cv. Marmolada smo vzgojili ukoreninjene rastlinice iz dolgotrajne in novo inicirane kulture te sorte in jih posadili v matični nasad. Vegetativne in generativne lastnosti njihovih hčerinskih rastlin smo primerjali z vegetativnimi in generativnimi lastnostmi standardno vzgojenih hlajenih sadik in rastlin, posajenih v poskus neposredno iz tkivne kulture. Med različnimi tipi sadik smo ugotovili statistično značilne razlike v številu živic na rastlino, številu cvetov na grm, številu plodov na grm in pridelku na grm. Največje razlike smo opazili v številu plodov na grm in pridelku na grm. Rastline, izvirajoče neposredno iz tkivne kulture, so imele skoraj enkrat večji pridelek v primerjavi s standardno vzgojenimi hlajenimi sadikami. Te so v številu plodov na grm in v pridelku na grm statistično značilno presegle hčerinske rastline matičnih rastlin, vzgojenih *in vitro*. Visoki pridelki rastlin, posajenih neposredno iz *in vitro* pogojev, so bili predvsem posledica njihovega močno podaljšanega zorenja.

Ključne besede: jagode, mikrorazmnoževanje, pridelek, kakovost, somaklonska variabilnost

Introduction

Strawberries can only be multiplied by vegetative methods, which explains the great dissemination of parasites. Most of strawberry producing countries thus have drawn up a program of certification to provide healthy planting material, which requires a minimum delay of 3 years before a healthy cultivar is at producer's disposal (Boxus & al. 1977). *In vitro* propagation of strawberries can speed up the propagation process. The possibility of somaclonal variation presents one of the drawbacks of this method. Mainly epigenetic changes such as hyperflowering, smaller fruit size and lower yields have been reported to occur. The quality of the material obtained *in vitro* and its behaviour in the field depends on genotype and tissue culture procedure (López-Aranda & al. 1994).

To determine the influence of tissue culture on field performance of cv. Marmolada, traditionally produced cold storage plants were compared with plants either derived directly from tissue culture or from micropropagated plants further propagated for one cycle in the nursery.

Materials and methods

Plant propagation

Meristems from terminal buds of dormant plants of cv. Marmolada, one of the main varieties cultivated in Slovenia, were isolated in December 1999 and grown *in vitro*. In the beginning of February 2000, newly developed shoots were multiplied according to the procedure described by Boxus & al. (1977). The same was done with shoots of cv. Marmolada grown *in vitro* over 3 years. Multiplied shoots originating from both newly established and old culture were transferred to a rooting medium for strawberries (Boxus & al. 1977). On 19th of April 2000 rooted shoots were transferred to soil. After 14 days of acclimatisation plants were transferred in larger pots and maintained in nethouse until beginning of August, when they were planted in the nursery. In spring 2001 runner plants were separated from mother plants and stored at -1° C until planting in the experimental field.

On 20th of June 2000 a new *in vitro* culture of cv. Marmolada was established from meristems excised from runner tips of field grown plants. Shoots were multiplied and rooted according to Boxus & al. (1977). On 1st of March 2001 rooted shoots were transferred to soil. After 14 days of

acclimatisation plants were transferred to larger pots and grown in the greenhouse until planting in the experimental field.

Field trial

On 22nd of May 2001 the field trial was planted on the Experimental Orchard of Agricultural Institute of Slovenia at Brdo near Lukovica. Four different plant types of strawberry cv. Marmolada were tested:

A. micropropagated plants originating from the *in vitro* culture established in June 2000,
B. runner plants from micropropagated mother plants, originating from the *in vitro* culture established in December 1999,
C. runner plants from micropropagated mother plants, originating from a culture maintained *in vitro* over 3 years,
D. conventionally produced cold storage plants of A quality.

A randomised block design with 4 treatments (plant types), 4 replications and 10 plants per plot was used. Following data were recorded: number of runners per plant, number of flowers per plant, number of fruits per plant and yield per plot. The STATGRAFICS Plus version 3.1 computer programme was used for statistical analyses. The analysis of variance was performed for the number of runners, number of flowers on 26th of July 2001, number of fruits and yield.

Results and discussion

Sanitary status of all plants included in the trial was very good. As expected, mutations were not observed, since propagation *in vitro* through axillary branching is generally considered to produce genetically stable material. Nevertheless, yellow leaf variants were reported to occur among micropropagated plants of strawberry cv. Redcoat by Nehra & al. (1994).

Considerable differences in vigour were observed among different plant types. Plants derived directly from tissue culture (plant type A) showed uniform and extremely vigorous growth with large leaves and very large flowers. Control plants (plant type D) were also uniform in vigour and had large leaves and flowers. In contrast, runner plants derived from micropropagated mother plants (plant type B and C) exhibited weak and variable growth and had small leaves and very small flowers.

Runner plants derived from micropropagated plants also produced the lowest number of flowers per plant as recorded on July 26th 2001 (Tab. 1). Plants of the type C had statistically significantly lower number of flowers per plant in comparison with plants derived directly from tissue culture as well as with traditionally propagated plants (Tab. 1). The number of flowers per plant of plant type B differed statistically significant only from plants obtained directly from tissue culture (Tab. 1). Since in strawberry not all of the flowers develop fruits, the number of fruits per plant was lower than the number of flowers recorded for all the plant types with exception of plants derived directly from tissue culture. The latter exhibited prolonged flower development. Consequently fruit ripening was also markedly prolonged. Fruits of plants derived directly from tissue culture were harvested from 4th of July until 24th of August whereas fruit ripening of other plant types ended more or less by 2nd of August. Plants derived directly from tissue culture gave statistically significant higher number of fruits and higher yields per plant than all other plant types including conventionally produced

cold storage plants mainly due to prolonged ripening (Tab. 1). In spite of long ripening time of type A plants there was very little variation in fruit number and fruit yield among picking dates. Fruit weight diminished only slightly towards the end of the ripening time.

Table 1: Vegetative and generative traits of different plant types of strawberry cv. Marmolada in the field

Plant type	Number of runners per plant	Number of flowers per plant	Number of fruits per plant	Yield per plant in g
A	10.1 a	13.4 a	16.15 a	97.6 a
B	7.0 c	8.1 bc	6.45 c	31.1 c
C	9.3 ab	7.3 c	6.25 c	30.9 c
D	7.6 bc	10.5 ab	9.0 b	54.2 b

Mean separation within column by Duncan's multiple comparison procedure
Values followed by a same letter do not differ significantly, $P < 0.05$

In the experiment of KARHU & HAKALA (2002) micropropagated plants of cv. Senga Sengana, planted directly in the field, also showed more abundant flowering and gave statistically significant higher marketable crop than control runner plants in the first cropping year. However no differences in yielding were recorded between micropropagated and control runner plants in cv. Zefyr (KARHU & HAKALA 2002). Similarly, an increased productivity of directly used micropropagated plants that was not accompanied by reduced weight was observed by NEHRA & al. (1994) for cv. Redcoat, whereas the yielding of cv. Veestar was not affected by the propagation method. In contrast to the results of KARHU & HAKALA (2002), SZCZYGIEŁ & al. (2002) recorded higher yields but diminished fruit quality of micropropagated plants of cvs. Senga Sengana, Kent and Dukat in comparison with the traditionally propagated runner plants. LÓPEZ-ARANDA & al. (1994) and SZCZYGIEŁ & al. (2002) quote other authors who observed a sharp decrease in average fruit weight, which was usually limited mainly to the plantlets coming directly from micropropagation. SZCZYGIEŁ & al. (2002) therefore suggested, that micropropagated plants should at least once be reproduced by runners before planting in the field. In our experiment runner plants derived from mother plants originating from either old or newly established *in vitro* culture both behaved poorly. The low vigour, flowering and yield of these plants were probably the result of the late planting of mother plants in the nursery and/or unfavourable weather condition during winter. As a consequence runner plants could not fully develop. Earlier planting of micropropagated plants in the nursery was prevented by poor development of rooted plantlets after acclimatisation due to extremely high temperatures.

Conclusions

Plants of cv. Marmolada derived directly from tissue culture showed markedly prolonged flower and fruit development. The number of fruits and yield of these plants were therefore almost twice as high as in conventionally produced cold storage plants, which showed statistically significantly better productivity than runner plants derived from micropropagated mother plants. The low vegetative and generative characteristics of the latter were probably the result of the late planting of mother plants in the nursery and/or unfavourable weather condition during winter.

Acknowledgement

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In vitro plant regeneration from somatic tissue of strawberry *Fragaria* x *ananassa* Duch.

In vitro regeneracija poganjkov v somatskem tkivu jagode *Fragaria* x *ananassa* Duch.

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Abstract. Successful shoot regeneration in somatic tissue is the basic requirement for *in vitro* induction of genetic variability as the new tool in plant breeding.

Somatic tissue excised from *in vitro* multiplied strawberry plants were tested on ability for plant regeneration. Leaves, petiole and stipules were inoculated on initial medium with BA and 2,4-D, or on medium with BA only after 1 hour pulse treatment with 2,4-D. Callus was induced on all sliced surfaces of explants inoculated on initial medium with growth regulators BA and 2,4-D during first 7 days of culture. Explants inoculated on initial medium with BA, after pulse treatment with 2,4-D did not develop callus but abundantly produced fenolic compounds, and tured necrotic in the first 24 hours.

Spontaneous plant regeneration was noticed on leaves explants with less developed callus tissue on initial medium with growth regulators during second week of culture. High percentage of explants with regenerated shoots were obtained after transfer on hormone-free medium.

The highest plant regeneration ability was in leaf tissue, less in petiole and stipules.

Callus induced in leaf tissue showed ability for constant plant regeneration during three months of culture and careful 4-week interval transfer on basal MS medium with 4.4¼M BA and 40 g/l sucrose.

Keywords: *in vitro*, somatic tissue, leaf explants, plant regeneration, strawberry, *Fragaria x ananassa* Duch., cv. Elsanta, cv. Marmolada

Izvleček. Uspešna regeneracija poganjkov v somatskem tkivu je osnovni pogoj za in vitro indukcijo genetske raznolikosti kot novega pristopa v žlahtnjenju rastlin.

Somatsko tkivo, pridobljeno iz in vitro razmnoženih rastlinic jagod, smo testirali glede sposobnosti za regeneracijo rastlin. Del izsečkov iz listov, pecljev in stipul smo prenesli neposredno na začetno gojišče z BA in 2,4-D, del pa predhodno 1 uro tretirali z 2,4-D in nato prenesli na gojišče s samo BA. Kalus se je induciral v prvih 7 dneh rasti kulture na rezani površini vseh izsečkov na gojišču z rastlinskimi hormoni BA in 2,4-D. Izsečki, predhodno tretirani z 2,4-D in inokulirani na gojišče z BA, niso razvili kalusa, ampak so proizvedli obilo fenolnih sestavin ter po 24 urah propadli.

Tekom drugega tedna rasti kulture na začetnem gojišču z rastnimi regulatorji BA in 2,4-D smo opazili spontano regeneracijo poganjkov na listnih izsečkih s slabo razvitim kalusnim tkivom.

Visok odstotek izsečkov z regeneriranimi poganjki smo pridobili po prenosu na gojišče brez rastnih regulatorjev.

Največjo sposobnost za regeneracijo poganjkov smo ugotovili pri listnem tkivu, slabšo pa pri tkivu pecljev in stipul.

Kalus, induciran v listnem tkivu, je pokazal sposobnost za stalno regeneracijo poganjkov tekom treh mesecev rasti kulture in po previdnem 4-tedenskem prestavljanju na bazalno MS gojišče z 4.4μM BA in 40 g/l sladkorja.

Ključne besede: in vitro, somatsko tkivo, listni izseček, regeneracija poganjkov, jagoda, *Fragaria x ananassa* Duch., cv. Elsanta, cv. Marmolada

Introduction

Development and application of reliable protocols for plant regeneration from tissue culture become the new tools for the improvement of strawberry (*Fragaria* x *ananassa* Duch.) cultivars.

Strawberry regeneration has been obtained *in vitro* from different somatic tissue: cell suspension (Damiano et al. 1995), leaves (Nehra et al. 1989; Nehra et al. 1990), petioles (Jones et al. 1988), stipules (Rugini and Orlando 1992, Jemmali et al. 1992), cotyledons (Miller and Chandler 1990), anthers (Rosati et al., 1975).

Because regeneration efficiency is strongly dependent on the cultivar, regeneration methods for strawberry shoots from nonmeristematic tissue have not been well defined yet.

The purpose of the present work was the evaluation of ability for *in vitro* shoot regeneration in strawberry somatic tissue.

Material and methods

Donor strawberry plants (*Fragaria* x *ananassa* Duch.) cv. Elsanta and cv. Marmolada, were propagated *in vitro* by protocol described by Boxus (Boxus et al. 1977).

Leaf strips, petioles and stipules were inoculated on basal MS medium with thiamine 0.4 mg/l, agar 8 g/l, pH 5.8. Four treatments were used: 1) BA 4.4¼M + 2,4-D 2.3¼M + 40 g/l sucrose; 2) BA 4.4¼M + 2,4-D 2.3¼M + 80 g/l sucrose; 3) BA 4.4¼M + 40 g/l sucrose; explants were under pulse treatment in MS liquid medium with 2,4-D 22.6¼M 1 hour prior inoculation; 4) is as (2) and also with pulse treatment as mentioned above.

Hundred and twenty leaf explants, 100 petiole explants of length 0.5 mm, and 60 stipule explants were inoculated for each treatment.

Explants with regenerated shoots were cultivated on MS hormone-free medium which promoted shoot elongation. Shoots grew in dense clusters, but it was possible isolated well developed plantlets. It was successfully aclimatized 194 plantlets of strawberry cultivar Elsanta.

Results

Regenerative ability was strongly influenced by genotype for three type of explants tested. Shoot regeneration from leaf, petiole and stipule explants in the strawberry cultivars Elsanta and Marmolada is presented in Table 1. Cultivar Elsanta showed better ability for callus initiation and shoot

regeneration in both treatments used. High percentage of leaf explants developed callus in the presence of BA and 2,4-D, and sucrose 40 g/l. Presence of 80 g/l sucrose suppressed callus development. Shoots which were initiated and regenerated on leaf explants cultured on initial medium, continued to grow on medium with BA 4.4¼M. It was possible to harvest regenerated plantlets from leaf explants during three months of culture, when explants with regenerative callus were transferring on fresh medium MS with BA every 4th week.

Petiole explants also developed callus and regenerated shoots, but in lower percentages than leaf explants.

In this experiment was not possible to obtain callus initiation and shoot regeneration in stipule explants of strawberry cv. Elsanta.

Induction of callus was very poor in leaf explants and petioles of cv. Marmolada. Stipule explants showed slightly better ability for shoot regeneration.

Pulse treatment with 22.6¼M 2,4-D on all three explant types of strawberry cultivars Elsanta and Marmolada caused abundant production of fenolic compounds and browning of plant tissue.

Discussion

The results show that ability of strawberry cv. Elsanta and cv. Marmolada to regenerate in vitro from somatic tissue (leaf, petiole, stipule) depends on the genotype. This fact also confirmed the results of experiments on cv. Redcoat and cv. Honeoye carried out by Nehra et al. (1988). Žebrowska et al. (2002) tested leaf and petiole of cv. Kama and clone B-302 on capacity for plant regeneraton, and also confirmed influence of the genotype. In their results petiole explants showed higher ability for regeneration, but higher number of regenerated plantlets per explant was in leaf tissue. We obtained better capacity for plant regeneration in leaf tissue of strawberry cv. Elsanta, and no ability for shoot regeneration in leaf and petiole tissue of cv. Marmolada. Only low percentage of stipule explants of cv. Marmolada regenerate shoots in our experiment. But Monticelli et al. (1995) reported on very high competence to regeneration from stipules of strawberry cv. Teodora and cv. Clea. They claimed also importance of 2,4-D as inducing factor in initial medium for callus induction, and reported similar as Jemmali et al. (1992) that BA is sufficient to induce shoot regeneration. We noticed that explants with callus induced on medium with BA 4.4¼M and 2,4-D 2.3¼M, continously regenerate shoots in leaf explants subsequently subcultured on medium with BA alone. Popescu et al. (1997) reported on the organogenetic potential of petiole-derived calli for a long period from at least 18 up to 29 weeks, but shoot formation was no longer recorded after 19 weeks.

In our experiment leaf explants of cv. Elsanta were able to regenerate shoots during 12 weeks.

Table 1: Shoot regeneration from leaf, petiole and stipule explants in two strawberry cultivars at two different treatments.

Cultivar/explant	Tretment	% callus regeneration explant^{-1}	% shoot regeneration explant^{-1}	Number of plantlets explant^{-1}
Elsanta				
Leaf	1	100	31.2	12*
	2	79.5	9.6	8*
Petiole	1	61.3	3.5	3
	2	20.8	2.8	3
Stipule	1	2.0	0	0
	2	0	0	0
Marmolada				
Leaf	1	3.5	0	0
	2	1.8	0	0
Petiole	1	1.2	0	0
	2	0	0	0
Stipule	1	1	1	3
	2	0	0	0

*shoots were harvested in vitro during 3 months

Conclusion

Shoot regeneration from somatic tissue (leaves, petioles, stipules) of strawberry (*Fragaria* x *ananassa* Duch.) is highly dependent on genotype.

2,4-D was essential for callus induction, while BA alone was later sufficient to induce shoot regeneration.

Regenerative calli induced on leaf explants of strawberry (*Fragaria* x *ananassa* Duch.) cv. Elsanta showed shoot regeneration ability during next three months in the presence of 4.4 ¼M BA.

Povzetek

Pri testiranju sposobnosti regeneracije jagod cvs. Elsanta in Marmolada iz različnih somatskih tkiv in vitro gojenih rastlin in učinkovitosti različnih načinov tretiranja s hormoni smo ugotovili:

* neučinkovitost tretiranja izsečkov z 2,4-D za 1 uro pred prenosom na gojišče z BA,
* velike razlike v sposobnosti regeneracije med proučevanimi kultivarjema,
* zelo slabo sposobnost regeneracije cv. Marmolada, pri kateri smo regeneracijo opazili samo pri 1 % izsečkov iz stipul,
* zelo dobro tvorbo kalusa in regeneracijo poganjkov cv. Elsanta iz izsečkov iz listov in nekoliko slabšo, a dobro regeneracijo iz izsečkov iz listnih pecljev
* neučinkovitost regeneracije iz stipul na preskušanih gojiščih,
* boljšo tvorbo kalusa oz. regeneracijo poganjkov na gojišču s 40 g/l saharoze v primerjavi z 80 g/l saharoze.

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NAVODILA AVTORJEM

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a) ZNANSTVENI ČLANEK je celovit opis originalne raziskave in vključuje teoretični pregled tematike, podrobno predstavljene rezultate z diskusijo in sklepe ter literaturni pregled: shema IMRAD (Introduction, Methods, Results And Discussion). Dolžina članka, vključno s tabelami, grafi in slikami, ne sme presegati 15 strani; razmak med vrsticami je dvojen. Recenzirata ga dva recenzenta.

b) PREGLEDNI ČLANEK objavi revija po posvetu uredniškega odbora z avtorjem. Število strani je lahko večje od 15.

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d) KONGRESNA VEST seznanja bralce z vsebinami in sklepi pomembnih kongresov in posvetovanj doma in v tujini.

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Teksti naj bodo pisani v angleškem jeziku, izjemoma v slovenskem, če je tematika zelo lokalna. Kongresne in društvene vesti so praviloma v slovenskem jeziku.

4. Naslov prispevka

Naslov (v slovenskem in angleškem jeziku) mora biti kratek, informativen in razumljiv. Za naslovom sledijo imena avtorjev in njihovi polni naslovi (če je mogoče, tudi štev. faxa in e-mail).

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Podati mora jedrnato informacijo o namenu, uporabljenih metodah, dobljenih rezultatih in zaključkih. Primerna dolžina za znanstveni članek naj bo približno 250 besed, za kratko notico pa 100 besed.

6. Ključne besede - Keywords

Število naj ne presega 10 besed; predstavljati morajo področje raziskave, obravnavane v članku. Člankom v slovenskem jeziku morajo avtorji dodati ključne besede v angleškem jeziku.

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Nanašati se mora le na tematiko, ki je predstavljena v članku ali kratki notici.

8. Slike in tabele

Tabele in slike (grafi, dendrogrami, risbe, fotografije idr.) naj v članku ne presegajo števila 10, v članku naj bo njihovo mesto nedvoumno označeno. Ves slikovni material naj bo oddan kot fizični original (fotografija ali slika). Tabele in legende naj bodo tipkane na posebnih listih (v tabelah naj bodo le vodoravne črte). Naslove tabel pišemo nad njimi, naslove slik in fotografij pod njimi. Naslovi tabel in slik ter legenda so v slovenskem in an–leškem jeziku. Pri –itiranju tabel in slik v besedilu uporabljamo okrajšave (npr. Tab. 1 ali Tabs. 1-2, Fig. 1 ali Figs. 1-2; Tab. 1 in Sl. 1).

9. Zaključki

Članek končamo s povzetkom glavnih ugotovitev, ki jih lahko zapišemo tudi po točkah.

10. Povzetek - Summary

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Uporabljene literaturne vire citiramo med tekstom. Če citiramo enega avtorja, pišemo ALLAN (1995) ali (ALLAN 1995), če sta dva avtorja (TRINAJSTIĆ & FRANJIĆ 1994), če je več avtorjev (PULLIN & al. 1995). Kadar navajamo citat iz večih del hkrati, pišemo (HONSIG-ERLENBURG & al. 1992, WARD 1994a, ALLAN 1995, PULLIN & al. 1995). V primeru, če citiramo več del istega avtorja, objavljenih v enem letu, posamezno delo označimo s črkami a, b, c itd. (WARD 1994a,b). Če navajamo dobesedni citat, označimo dodatno še strani: TOMAN (1992: 5) ali (TOMAN 1992: 5–6). Literaturo uredimo po abecednem redu, začnemo s priimkom prvega avtorja, sledi leto izdaje in naslov članka, mednarodna kratica za revijo (časopis), volumen poudarjeno, številka v oklepaju in strani. Npr.:

HONSIG-ERLENBURG W., K. KRAINER, P. MILDNER & C. WIESERR 1992: Zur Flora und Fauna des Webersees. Carinthia II **182/102** (1): 159–173.

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Knjige, poglavja iz knjig, poročila, kongresne povzetke citiramo sledeče:

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Manuscripts submitted for publication in *Acta Biologica Slovenica* should not contain previously published material and should not be under consideration for publication elsewhere.

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Articles and notes should be submitted in English, or as an exception in Slovene if the topic is very local. As a rule, congress and association news will appear in Slovene.

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Titles (in Slovene and English) must be short, informative, and understandable. The title should be followed by the name and full address of the author (and if possible, fax number and e-mail address).

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The abstract must give concise information about the objective, the methods used, the results obtained, and the conclusions. The suitable length for scientific articles is approximately 250 words, and for brief note articles, 100 words.

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There should be no more than ten (10) keywords; they must reflect the field of research covered in the article. Authors must add keywords in English to articles written in Slovene.

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The introduction must refer only to topics presented in the article or brief note.

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Articles should not contain more than ten (10) illustrations (graphs, dendrograms, pictures, photos etc.) and tables, and their positions in the article should be clearly indicated. All illustrative material should be provided as physical originals (photographs or illustrations). Tables with their legends should be submitted on separate pages (only horizontal lines should be used in tables). Titles of tables should appear above the tables, and titles of photographs and illustrations below. Titles of tables and illustrations and their legends should be in both Slovene and English. Tables and illustrations should be cited shortly in the text (Tab. 1 or Tabs. 1-2, Fig. 1 or Figs. 1-2; Tab. 1 and Sl. 1).

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Articles shall end with a summary of the main findings which may be written in point form.

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11. Literature

References shall be cited in the text. If a reference work by one author is cited, we write ALLAN (1995) or (ALLAN 1995); if a work by two authors is cited, (TRINAJSTIĆ & FRANJIĆ 1994); if a work by three or more authors is cited, (PULLIN & al. 1995); and if the reference appears in several works, (HONSIG-ERLENBURG & al. 1992, WARD 1994a, ALLAN 1995, PULLIN & al. 1995). If several works by the same author published in the same year are cited, the individual works are indicated with the added letters a, b, c, etc.: (WARD 1994a,b). If direct quotations are used, the page numbers should be included: TOMAN (1992: 5) or (TOMAN 1992: 5–6).

The bibliography shall be arranged in alphabetical order beginning with the surname of the first author followed by the year of publication, the title of the article, the international abbreviation for the journal (periodical), the volume (in bold print), the number in parenthesis, and the pages. Examples:

HONSIG-ERLENBURG W., K. KRAINER, P. MILDNER & C. WIESER 1992: Zur Flora und Fauna des Webersees. Carinthia II **182/102** (1): 159–173.

TRINAJSTIĆ I. & J. FRANJIĆ 1994: Ass. *Salicetum elaeagno-daphnoides* (BR.-BL. et VOLK, 1940) M. MOOR 1958 (*Salicion elaeagni*) in the Vegetation in Croatia. Nat. Croat. **3** (2): 253–256.

WARD J. V. 1994a: Ecology of Alpine Streams. Freshwater Biology **32** (1): 10–15.

WARD J. V. 1994b: Ecology of Prealpine Streams. Freshwater Biology **32** (2): 10–15.

Books, chapters from books, reports, and congress anthologies use the following forms:

ALLAN J. D. 1995: Stream Ecology. Structure and Function of Running Waters, 1st ed. Chapman & Hall, London, 388 pp.

PULLIN A. S., I. F. G. MCLEAN & M. R. WEBB 1995: Ecology and Conservation of *Lycaena dispar*: British and European Perspectives. In: PULLIN A. S. (ed.): Ecology and Conservation of Butterflies, 1st ed. Chapman & Hall, London, pp. 150–164.

TOMAN M. J. 1992: Mikrobiološke značilnosti bioloških čistilnih naprav. Zbornik referatov s posvetovanja DZVS, Gozd Martuljek, pp. 17.

12. Format and Form of Articles

Articles should be send as Microsoft Word document (doc) or rich text format (rtf) using "Times New Roman CE 12" font with double spacing, align left and margins of 3 cm on A4 pages. Paragraphs should be separated with an empty line. The title and chapters should be written bold in font size 14. All scientific names must be properly italicized. Used nomenclature source should be cited. Tables and illustrations shall accompany the texts separately. The original manuscript, two copies, and an electronic copy on a 3.5" computer diskette, on CD-ROM or by e-mail must be given to the editor-in-chief. All articles must be proofread for professional and language errors before submission.

13. Peer Review

All Scientific Articles shall be subject to peer review by two experts in the field (one Slovene and one foreign) and Brief Note articles by one Slovene expert in the field. Authors may nominate a foreign reviewer in an accompanying letter. Reviewed articles accepted for publication shall be corrected by the author. Authors shall receive fifty (50) free copies of the journal upon publication. In the event an article is rejected, the original material shall be returned to the author together with the negative determination of the editor-in-chief.

ČLANKI – ARTICLES: