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Induction of multi-nucleate microspores in anther culture of *Salix viminalis* L.

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Abstract: The aim of the work was to determine how the induction of androgenesis in selected genotypes of *Salix viminalis* is affected by thermal factors and medium composition. Anthers isolated from male clones of *S. viminalis* were pre-cultured at 4, 27 and 32°C for two to eight days in liquid Kyo medium with and without the addition of mannitol and on solid MS or WPM medium. The solid media were supplemented with different concentration of disaccharides and various combinations of growth regulators including kinetin, 2iP, IAA and IBA. Multi-nucleate microspores indicative of androgenesis were observed in anthers pre-cultured for seven days at 4°C in liquid Kyo medium containing mannitol. The rate of androgenesis was higher when the anthers were transferred to solid modified MS medium containing high concentrations of sucrose and kinetin. In studied genotypes of basket willow early uninucleate microspores underwent sporophytic divisions. Ultrastructural observations showed differences in cellular arrangement of pre-stressed microspores. In *Salix viminalis*, microspores without starch grains and decreased number of lipid bodies were potentially androgenic cells.

Additional key words: mannitol, thermal shock, TEM, stress response

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Introduction

Androgenesis is one of the most effective and widely used methods for producing haploid plants in vitro. The somatic cells of the resulting plants have the chromosome number as the gametes. Doubling the chromosomes number produces homozygotic dihaploid plants, which are valuable in research on plant breeding, genetics and development (Touraev et al. 1997; Forster et al. 2007). Dihaploid plants can be used to breed new plant varieties, or to speed up selection for heterosis, disease resistance, and tolerance to biotic or abiotic stresses (Germana 2006).

Research on androgenesis have focused on species of economic or ecological importance. Anther culture has been used to produce haploid plants of fruit trees such as: *Citrus* sp, *Vitis* sp *Litchi* sp and *Carica papaja* (Ochatt and Zhang 1996), ornamental trees *Peltophorum pterocarpum*, medical trees: *Aesculus hippocastanum*, *Azadirachta indica* and species cultivated for great economical and ecological value as *Coffea arabica*, *Hevea brasiliensis, Quercus petrea*, and several species and hybrids of the genus *Populus* (Strivastava and Chaturvedi 2008). Among woody trees, the species on which the most research has been carried out are those belonging to the genus *Populus*. An effective and reliable method for inducing and regenerating haploid plants of this genus has been developed (Kiss et al. 2001). In spite of the many attempts to induce androgenesis in woody species the list of investigated species is limited and a lot of significant trees have not been studied yet.

To our knowledge no research on androgenesis has been carried out on *Salix* species. *S. viminalis*, is one of the economically most important species and is cultivated on a wide scale for biomass. It is also used in phyto-remediation because of its capacity to extract heavy metals from contaminated soil (Pulford and Watson 2003).

In studies on induction androgenesis, many factors that switch microspore from the gametophytic to the sporophytic development have been examined. The one of symptoms of androgenic development is multi-nucleate and multicellular stage of microspores (Rodrigues et al. 2005).

Genotype of the mother plant play a major role in pollen embryogenesis induction by in vitro anther culture. Androgenic response of microspores can vary between genera, species, and even varieties of the same species with different levels of ploidy (Zenkteler 1984).

The induction of androgenesis is also greatly affected by the physiological state of the donor plant. The proportion of microspores that give rise to embryos or callus tissue is higher in anthers collecting from plants at the beginning of the flowering cycle (Smykal 2000). Furthermore, the induction of androgenesis is affected by the physical conditions under which the donor plants were cultivated, including temperature, light intensity, and the length of the photoperiod (Palmer and Keller 1997). Pre-cultures of parental plants or explants were recommended for many herbaceous species. In woody plants controlled growth of donor plants in growth chambers is practically impossible and the experiments are carried out on material collected from the natural environmental conditions (Baldurson and Ahuja 1996).

In tree species, sporophytic nuclear division in microspore cultures can be induced by stress-inducing procedures such as cold and heat pre-treatments, carbohydrate starvation or nitrogen deprivation (Hosp et al. 2007). These procedures can be carried out on either whole flower buds or explants (anthers or microspores).

The chemical composition of the culture medium, especially the combination and the concentrations of the sugar and growth regulators have profound effect on embryonic response of microspores. As of yet, however, no unified medium that induces androgenesis has been devised. Among the basal media that have been used to induce androgenesis in different tree species are: MS (Murashige i Skoog 1962); M (Miller 1965); N (Nitsch 1972) and N6 (Chu 1978). These media can be supplemented with different concentrations and combinations of growth regulators and other chemical factors (active charcoal, glutamine, natural extracts etc.) that affect plant development (Ochatt and Zhang 1996; Strivastava and Chaturvedi 2008.).

The aim of the present study was to determine how the induction of androgenesis in selected clones of *Salix viminalis* is affected by abiotic factors and the medium composition. Microspore morphology and ultrastructure were observed during the pre-culture and culture periods using fluorescent microscopy and transmission electron microscopy.

Materials and methods

Plant material and anthers preparation

The study was carried out using shoots cut from plants of six male clones (2, 7, 10, 14, 37 and 72) of *S. viminalis* growing in the field of the Institute of Plant Genetics Polish Academy of Science in Poznań. The four years old donor plants were grown outdoors in unprotected plots exposed to natural atmospheric conditions. The shoots were cut at three week intervals from the beginning of January to the middle of March. The collected branches were transferred to the laboratory, where they were placed in containers with water and kept at a temperature of 22°C ± 2°C under natural lighting. The experiments were carried out in two years.

Catkins with anthers containing immature pollen grains at different stages of development (from the tetrad to the uninucleate microspore stage) were selected (see section on cytological analysis). Inflorescence with anthers containing microspores at the early uninucleate stage predominated. After dissection the catkins were surface disinfected for four minutes in a solution containing sodium hypochlorite with a concentration of available chlorine of 5% and rinsed three times in sterile water.

Pre-culture and culture of anthers

In order to determine the effect of temperature, osmotic pressure, and medium composition on microspore development, isolated anthers were pre-cultured in Petri dishes containing 5 ml of filter-sterilized liquid Kyo medium (Kyo and Harada 1986). Petri dishes with anthers were then kept in the dark at either 4, 27 and 32°C for two to eight days. Two variations of this medium were used: with mannitol (0.4M), and without (Fig. 1). Following the pre-treatments, anthers were transferred to different solid media (Table 1) and kept in a culture room at 21±2°C.

Part of the anthers were not pre-cultured on liquid Kyo medium, but were placed directly on different solid culture media (Table 1). The cultures then were pretreated at temperatures of 4, 27 or 32°C. Every day for eight days, part of the pre-cultures from each of the incubators were transferred to the culture room.

Anthers cultured continuously on solid media (Table 1) in the dark in the culture room were regarded as the control.

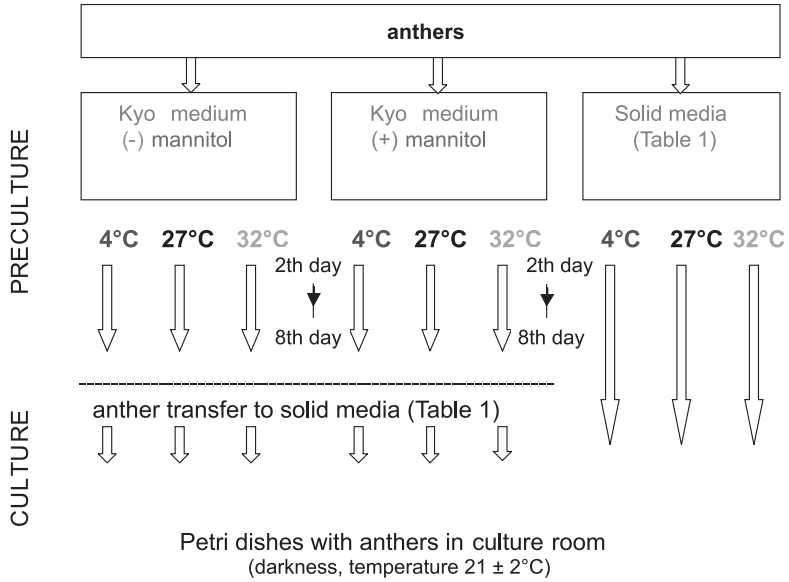

Fig. 1. Diagram of *S. viminalis* anther pre-cultures and cultures

The solid media used were modified MS (Murashige and Skoog 1962) or WPM (Lloyd and McCown 1981) medium. The media were solidified with Difco agar or Phytagel and supplemented with the following cytokinins: kinetin (Kin), N^6-(3-methyl-but-2-enyl)-adenine (2iP) and auxins: indole-3-acetic acid (IAA), indole-3-butyric acid (IBA) (Fig. 1; Table 1). The pH of the medium was adjusted to 5.8 before autoclaving at 1×10^5 Pa and 121°C for 20 min.

Twenty five anthers were placed on each dish with the long axis parallel to the surface of the medium. Altogether, 9725 anthers were cultured. All variations were run in triplicate.

Table 1. Solid medium variants used for anther cultures of *S. viminalis*

Medium	Growth regulators [mg l^{-1}]		Modifications [g l^{-1}]	Additives [mg l^{-1}]
MS	Kin 8.0	IBA 0.5	sucrose 50	
			maltose 40	
	Kin 6.0	IBA 0.5	sucrose 100	activated charcoal 1000
	Kin 8.0	IAA 0.5	sucrose 100	
	Kin 6.0	IAA 0.5	sucrose 100	
	Kin 6.0	IAA 0.5	sucrose 100	activated charcoal 1000
	Kin 6.0	IAA 0.5	sucrose 100 myo-inozytol 0.05	Pluronic F-68 100
	Kin 6.0	IAA 0.5	phytagel 3	glutamine 300
	Kin 6.0	IAA 0.5	maltose 60 phytagel 3	
WPM	Kin 6.0	IAA 0.5	maltose 60 phytagel 3 myo-inozytol 0.05	activated charcoal 1000

Cytological analysis

Anthers were selected on the basis of the stage of microspore development. It was determined by examining anthers from the basal, central and apical part of the catkins under a fluorescent microscope. Anthers were placed on a glass microscopy slide in a drop of aqueous solution of DAPI (4,6-diamidino2 phenylindol dichloride). The anthers were then squashed with a cover slide and examined under an OPTON Axioscope fitted with a Zeiss MC80DX microscopic camera using Fuji Film 100.

Table 2. Multi-nucleate microspores of *S. viminalis* induced on solid media after four weeks of cultures

	Pretreatments		Length of pre-treatment [days]	Culture medium [mg l^{-1}]
Clone 72	MS*	4°C	8	*MS Kin 6.0 IAA 0.5 sucrose 100000
	Kyo (–)	4°C	6	
		32°C	6	
		32°C	7	
		27°C	6	
		27°C	7	
		27°C	8	
Clone 14	Kyo (+)	4°C	6	**MS Kin 8.0 IBA 0.5 sucrose 50000 maltose 40000
		4°C	8	
		32°C	6	
	Kyo (–)	4°C	7	
		27°C	6	
		27°C	7	
		27°C	8	
	MS**	32°C	7	
	MS**	32°C	8	

Cultured anthers were examined every day during the eight-day pre-culture period, and after four and eight weeks of culture on solid medium.

Ultrastructural analysis

Initial anthers and anthers collected from liquid pre-cultures were fixed overnight at 4°C in a mixture of para-formaldehyde and glutaraldehyde (final concentration of 2%) in 0.05 M cacodylic buffer (pH 6.8). The anthers were then rinsed three times in pure cacodylic buffer, post-fixed for two hours at 4°C in 1% osmium tetroxide, and counter-stained with 2% aqueous uranyl acetate (pH 5.0). Subsequently, the samples were dehydrated in graded acetone series and embedded in epoxy resin as described by Spurr (1969). Ultra-thin sections (90 nm) were counter-stained for twenty minutes in lead citrate. The sections were examined using a JEOL JEM 1200 EXII transmission electron microscope (TEM) at acceleration of 80 kV.

Results

Characteristic of initial microspores

Majority of the anthers selected for the study contained microspores in the early uninucleate stage with centrally located nucleus (Fig. 2A). The spherical nucleus and the individual nucleoli were intensely stained by the fluorochrome DAPI. The cytoplasm contained numerous vacuoles with electron-dense material, as well as ellipsoidal mitochondria with electron-translucent areas. There were also abundant amyloplasts of various shapes containing numerous starch grains (Fig. 2B). Electron-dense bodies, most

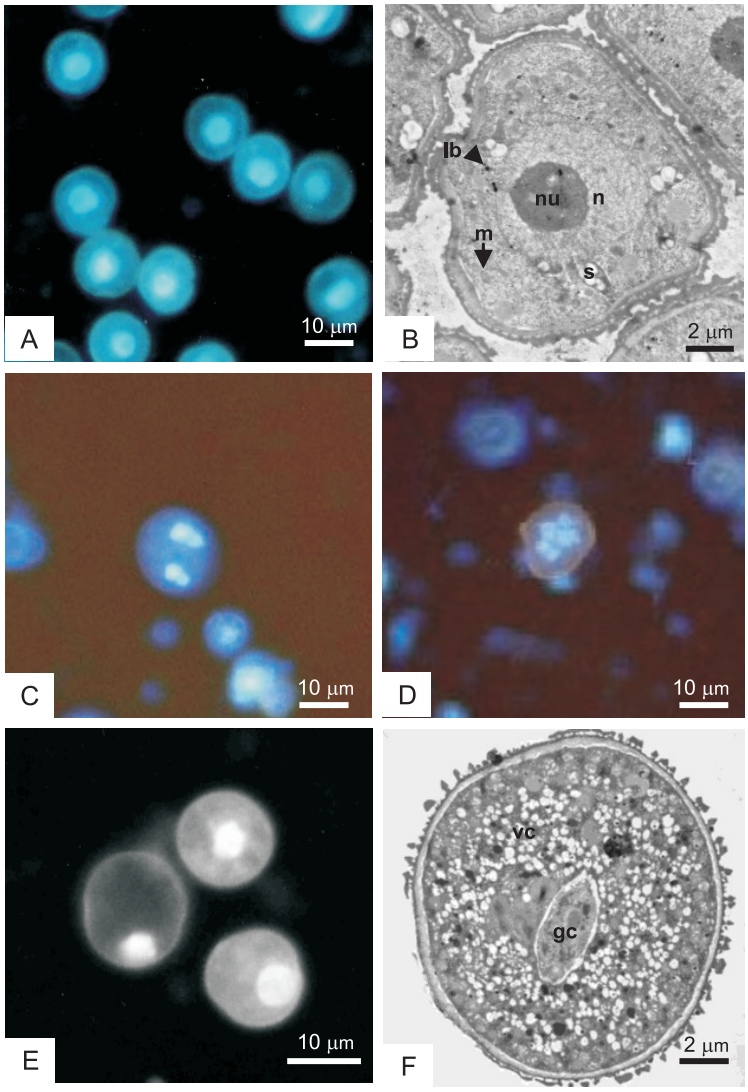

Fig. 2. Microspores and pollen grain of *Salix viminalis* in cytological and ultrastructural observations. A (FM) and B (TEM) – the initial early-uninucleate microspores; C (FM) – the tetranucleate microspore and D (FM) – the pentanucleate microspore after 7 days of mannitol and cold pretreatment; E (FM) – microspores after 8 days of heat pretreatment; F (TEM) – the pollen grain with vegetative (vc) and generative cell (gc)

n – nucleus, nu – nucleolus, m – mitochondrion, s – starch grain, lb lipid body

likely lipid bodies were associated with the endoplasmic reticulum and the Golgi apparatus, which had expanded internal membrane structure.

Cytology of microspores during pre-culture

In microspores pre-cultured in liquid medium at 4°C, the position of the nucleus was the same as in the initial microspores (Fig. 2A). The nucleus was spherical and centrally located within the cells.

In microspores pre-cultured in liquid medium at 27°C, the viability of microspores decreased throughout the pre-culture period. The nuclei migrated to the periphery of the cell, and there were changes in the shape of the cell as a whole (not shown).

Microspores stressed at 32°C were irregular in shape and some of them were not stained with DAPI. The formation of enlarged microspores were noticed (not shown). The formation of swollen microspores range between 20 and 50% percent of the total, depending on the length of the pre-culture period.

At 27 and 32°C, nuclear division was not affected by the presence or absence of mannitol in the medium or by the genotype of the donor plant.

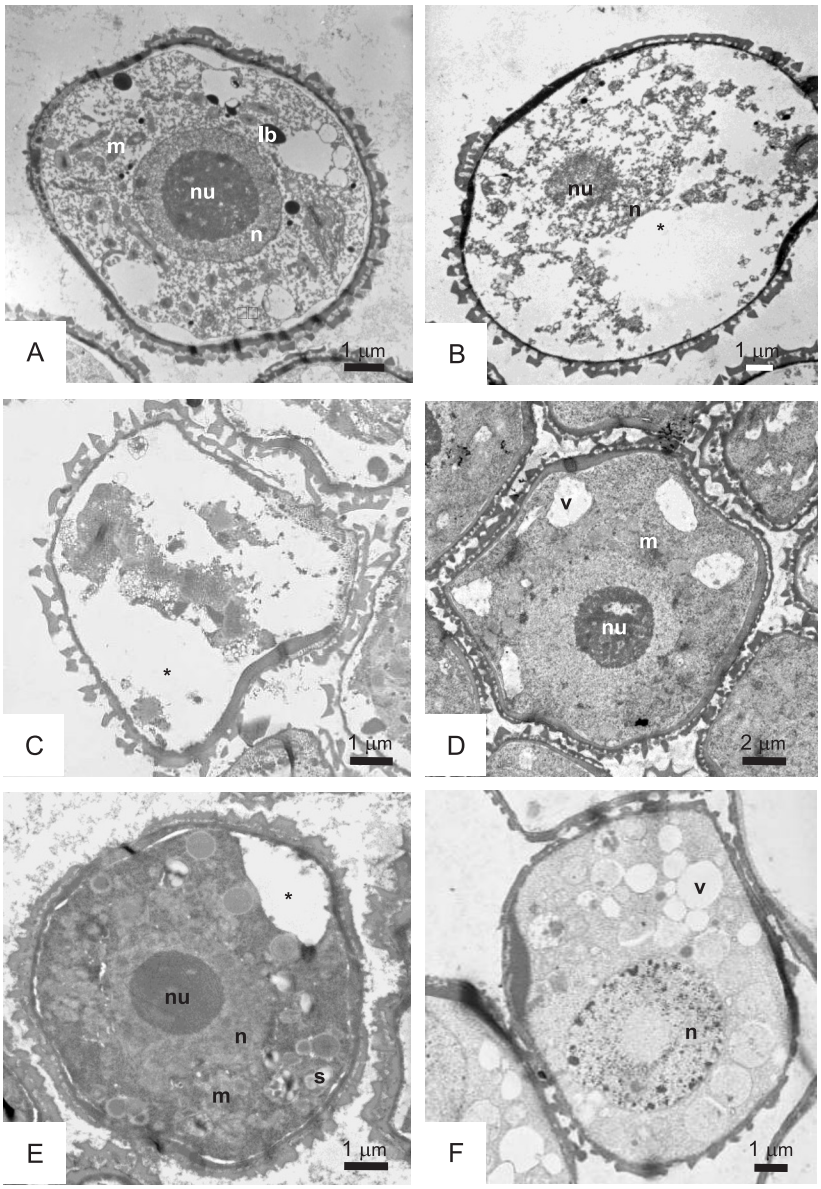

Fig. 3. TEM observations of *S. viminalis* microspores pre-cultured at 27°C and Kyo medium: with mannitol (A–C) and without mannitol (D–F). A – a microspores with abundant mitochondria and enlarged nucleus located centrally in rarefied cytoplasm; B – a microspores containing rarefied cytoplasm with clear areas and periphery situated nucleus; C – degenerating microspore with remnants of cytoplasm; D – a microspore without starch grains with vacuoles and mitochondria; E – the microspore showing cellular arrangement of initial microspores with the exception of clear area presented in cytoplasm; F – enlarged periphery located nucleus in rarified cytoplasm of a microspore.

n – nucleus, nu – nucleolus, m – mitochondrion, s – starch grain, lb lipid body, v – vacuole, *– clear area in cytoplasm

Multi-nucleate microspores were seen in pre-cultures of clone 14 in medium containing mannitol at 4°C, after seven days of stress treatment (Fig. 2C-D). The first androgenic division was probably symmetric, based on the uniform size and staining intensity of the daughter nuclei (Fig. 2D).

In anthers pre-cultured on solid medium at 4°C, microspores appeared to be unchanged and the spherical nucleus was centrally located as in initial microspores (Fig. 2A).

In microspores pre-cultured on solid media at 27 and 32°C, the nucleus in some of the microspores had migrated to the periphery by the eighth day of the pre-culture period (Fig. 2E). In some cases, developing and even germinating pollen grains were observed. Mature gametophytes consisted of two cells (Fig. 2F). On solid media no multi-nucleate microspores were observed during the eight-day of pre-culture period.

Ultrastructure of microspores during pre-culture

In anthers pre-cultured in liquid medium at 27 and 32°C, microspore ultrastructure from six to eight days of pre-culture depended on whether the medium contained mannitol or not.

In the absence of mannitol, three types of microspores were observed. The first type had a spherical shape and a round nucleus that was sometimes enlarged. The nucleus with large nucleolus was centrally placed and surrounded by rarefied cytoplasm. The cytoplasm contained abundant mitochondria, vacuoles, and a few amyloplasts with two to four starch grains. In cytoplasm electron-dense bodies were also observed (Fig. 3A).

The second type had a peripheral nucleus (Fig. 3B). The cytoplasm was rarefied, and contained fewer vacuoles, mitochondria and amyloplasts. The amyloplasts contained only single starch grains.

In the third type of microspores the cytoplasm was very rarefied, with extensive electron-translucent spaces (Fig. 3C). These features indicated that the cells were dead. The cytoplasm contained scant mitochondria and numerous tiny vacuoles.

In the presence of mannitol, also three types of microspores were observed but they were different from those previously described. The first and most common type had a round nucleus that was centrally placed. Individual nucleolus was visible. The cytoplasm contained no amyloplasts or lipid bodies, and only scant vacuoles (Fig. 3D).

The second type represented microspores at the early uninucleate stage. The dense cytoplasm contained a lot of amyloplasts with single spherical starch grains. In some amyloplasts, the starch grains were elongated, which indicates that starch was being released. These immature pollen grains contained numerous spherical mitochondria and electron-dense bodies (Fig. 3E).

The third type, approximately 15% of the observed microspores had an enlarged peripheral nucleus with a single nucleolus. The cytoplasm was rarefied and contained mitochondria and numerous vacuoles (Fig. 3F).

Effect of pretreatments and medium compositions on microspores

After four weeks of culture on solid media, multi-nucleate microspores were observed in anthers of clones 14 and 72. Androgenic divisions of microspores were induced in anthers after temperature and/or mannitol pretreatments (Table 2). Nuclear divisions in clone 14 and 72 were observed in those cultures pretreated for six or eight days at temperatures of 4, 27 and 32°C. Mannitol treatment to the anthers induced microspore nucleus divisions only in clone 14 (Table 2). Microspores of clone 14 underwent nuclear divisions on medium containing 8.0 mgl^{-1} kinetin and the disaccharides maltose and sucrose. On the other hand, microspores of clone 72 underwent nuclear division on media containing 6.0 mgl^{-1} kinetin and sucrose (Table 2).

Discussion

The induction and course of androgenesis is affected by many internal and external factors. Internal factors include the genotype and the developmental stage of the microspores. External factors include pre-culture treatments of explants (temperature and osmotic pressure), culture condition and medium composition (Heberle-Bors 1998). In the present study, conditions for pre-culture and culture were selected on the basis of previously published results of experiments on tree species.

Induction of androgenesis is highly dependent on genotype (Touraev et al. 1997). In the present study, the induction of androgenesis depended on the genotype tested. Androgenesis was induced only in clones 14 and 72.

In the present study, the induction of sporophytic divisions in gametophytic cells depended on temperature, osmotic pressure and the developmental stage of the microspores tested. In tree species, the switch of the gametophytic development to the androgenic pathway has been induced at stage of uninucleate microspore (tetrad phase and the first mitotic phase) (Baldursson and Ahuja 1996). In same woody species, the period during which androgenesis can be induced is expanded. In *Sorbus domestica*, for example, this period included the tetrad phase and the uninucleate microspore stage (Arrillaga et al. 1995). In *Albizzia lebbeck* and *Olea europaea*, it included the

uninucleate and binucleate microspore stages (Gharyal et al. 1983; Bueno et al. 2005).

In other species, on the other hand, androgenesis can be induced only during a more restricted period. In *Aesculus hippocastanum*, androgenesis was induced only in uninucleate microspores at a particular phase of development when the nucleus was in a central position (Radojevic 1991). In *Cocos nucifera* and *Citrus clementina*, androgenesis was induced only during the late uninucleate stage of microspore development (Perera et al. 2008; Ramirez et al. 2003).

During the first year of the present studies, the anthers used contained post meiotic cells at different stages of development, from the tetrad phase to the uninucleate microspore phase. It was found that sporophytic divisions of microspores were induced only during the early uninucleate stage and was impossible to induce androgenesis during the tetrad phase even with pre-culture. Microspores at the bi-nucleate stage were also resistant to androgenesis and continued developing as programmed into mature pollen grains. In the second year, therefore, only anthers at the early uninucleate stage were used.

Shifting development from the gametophytic to the sporophytic pathway usually requires pre-culture under various stressful conditions (Wang et al. 2000). In *Citrus clementina*, pre-culture at low temperatures for 48 hours induces mitotic nuclear division, giving rise to two nuclei of the same size, after which embryonic development commenced. During this process, the microtubular cytoskeleton was disrupted (Germana and Chiancone 2003). In anther cultures of *Citrus madurensis*, the formation of callus tissue and structures similar to embryos was possible only after pre-culture at 3°C for five to ten days (Chen, 1985). In *Pyrus pyrifolia*, pre-culture at 5°C for ten days induced androgenic callus formation and increased the proportion of anthers that produced callus from 9.6% to 85.8% (Kadota and Niimi 2004). Cold pretreatment conditions increases embryonic potential in explants after two or three days in *Malus domestica*, or after four days in *Populus maximowiczii* (Höfer 2004; Stoehr and Zsuffa 1990). In *Populus* hybids, however, pre-culture for long periods reduced microspore viability (Deutsch et al. 2004).

In the present study, anthers were pre-cultured from two to eight days at 4°C. There was no loss in viability during this period, although microspores did not undergo sporophytic division.

The potential for androgenesis can be increased by exposing explants to two different kinds of stress, such as osmotic stress in combination with high or low temperatures (Maraschin et al. 2005). In *Hordeum vulgare*, osmotic stress in combination with low temperatures increased the rate of androgenesis from 1% to 8.5% (Wang et al. 2000). In our study, the presence of mannitol in the pre-culture medium preserved microspore viability. Pre-culture in medium containing mannitol at 4°C effectively induced nuclear division and the formation of multi-nucleate microspores.

Osmotic stress in combination with high temperatures can also induce androgenesis. In *Malus domestica*, exposure of microspores to osmotic stress and mild heat shock (27°C) induces sporophytic division within two to five days. Pre-culture at 33°C induces pollen embryogenesis after five days in *Quercus suber*, and after one day in *Olea europaea* (Bueno et al. 1997; Bueno et al. 2005).

In the present study, pre-culture in liquid medium containing mannitol at 27 and 32°C did not induce the formation of multi-nucleate microspores during the pre-culture period. Multi-nucleate microspores did develop, however, when the anthers were transferred to solid medium. Examination of the pre-cultures revealed enlarged microspores that were similar to those observed in other species such as *Malus domestica* and *Olea europaea* (Höfer et al. 1999; Höfer 2004; Bueno et al. 2005). These microspores are considered to have embryogenic potential, and it is probably these microspores that underwent nuclear division when the anthers in the present study were transferred to solid medium containing growth regulators.

TEM observations revealed that mannitol inhibited the development of early multi-nucleate microspores in mature pollen grains without reducing their viability.

Under stress, microspores enlarge, and their cytoplasm undergoes structural reorganization (Touraev et al. 1997). In the present study, pre-culture in medium containing mannitol induced the formation of characteristic enlarged cells with scant amyloplasts and liposomes and abundant vacuoles. This agrees with a previous study on *Malus domestica* (Höfer et al. 1999). Microspores cultured under stressful conditions often show signs of degradation of cellular components as a such as a decrease in the number of amyloplasts, lipid bodies and ribosomes (Hosp et al. 2007). These microspores are capable of switching from their normal gametophytic developmental pathway, giving rise to androgenic embryos or callus. In the present study, microspores with a large peripheral nucleus surrounded by diffuse cytoplasm were also seen after pre-culture in medium with mannitol at 27 and 32°C. This is not characteristic of microspores developing along the gametophytic pathway, and indicates that a switch to the sporophytic pathway had occurred (Ramirez et al. 2001).

The choice and concentration of the carbohydrate source used in the culture medium has a significant effect on embryogenesis in microspores (Indrianto et al. 1999). In herbaceous plants with tree-celled pollen grains, androgenesis can be induced on media con-

taining a high sugar concentration. In herbaceous plants with two-celled pollen grains, microspores switch to the sporophytic pathway on medium with a low sugar concentration (Baldurson and Ahuja 1996). In woody plants with three-celled pollen grains, androgenesis has been successfully induced in *Hevea brasiliensis*, *Ulmus americana* and *Azidirachta indica* (Gautam et al. 1993; Chen et al. 1982; Redenbaught et al. 1981). Most reports of successful induction of androgenesis in woody plants deal with species that have two-celled pollen grains, such as *S. viminalis* (Fig. 2f). In woody trees media with high concentrations of sucrose have been used to induce androgenesis in *Populus×simonigra*, *Malus pumilia*, *Citrus microcarpa* and *Cocos nucifera*, as well as in the species with three-celled pollen grains listed above (Baldursson and Ahuja 1996; Perera et al. 2008). In the present study, multi-nucleate microspores were observed on medium containing relatively high concentration of sucrose (100 g l^{-1}).

In *Populus maximowiczii* and *P. trichocarpa*, androgenesis was induced on medium supplemented with 60 g l^{-1} maltose (Baldursson et al. 1993). In some cereal genotypes, nuclear divisions in microspores do not take place on media containing sucrose, but does take place when maltose is added to the medium (Palmer and Keller 1997). In the present study, the use of medium containing both sucrose and maltose seems to have been an essential factor in inducing nuclear division in clone 14.

Other supplements have also been used in various studies. For example, glutamine and activated charcoal improve regeneration in *Poncitrus trifoliata* and *Cocos nucifera* (Germana 2006; Perera et al. 2008). These supplements had no effect on nuclear division in the present study. Nuclear division was also not affected by reducing the concentration of myo-inositol, by adding Pluronic F-68 or Phytagel to the medium.

In the present study, multi-nucleate microspores were observed in pre-cultured anther cultures. The formation of multi-nucleate microspores was strongly affected by conditions during pre-culture, such as osmotic stress and thermal conditions. It was also affected by the concentrations of certain components of the culture medium, especially disaccharides, kinetin, and the auxins IAA and IBA. It was difficult to determine which factor was actually responsible for inducing androgenesis.

Microspores of tree species are not easily switched from the gametophytic pathway to the sporophytic pathway. The successful induction of the formation of multi-nucleate microspore in the present study therefore represents an important step in research on inducing androgenic regeneration in *Salix* species.

Conclusions

Multi-nucleate microspores were observed in anthers pre-cultured for seven days at 4°C in liquid Kyo medium containing mannitol. This was especially true for clone 14. The rate of androgenesis was higher when the anthers were transferred to solid modified MS medium containing kinetin and auxin and cultured in the dark at 21±2°C. Mannitol inhibited the development of early uninucleate microspores in mature pollen grains, thereby affecting the induction of androgenesis.

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