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Regulation of cytokinin response-competence by cold treatment of mature *Pinus sylvestris* tissues *in vitro*

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Abstract

One of the possible reasons for lack of morphogenesis during tissue culture of mature tissues is the inability to respond to cytokinin. In the present experiments, cold incubation of pine (*Pinus sylvestris* L.) bud explants was used as a means to affect cytokinin response competence. Pulse treatment with cytokinins of pine bud explants cultivated at 22 °C did not result in increased morphogenesis. New bud and shoot formation was achieved on pine bud explants which were pulse-treated with BAP in combination with cold incubation. It is concluded that, due to the temporary nature of juvenilization of mature pine bud explants, it is necessary to combine both physical and biochemical means to promote morphogenic competence and to achieve further development in tissue culture.

Key words: cold incubation, cytokinin response competence, morphogenesis *in vitro*, *Pinus sylvestris*.

Introduction

Micropropagation is the most effective vegetative reproduction technique permitting rapid propagation and distribution of elite genotypes. Micropropagation of woody species with mature tissues could be very valuable, but it is often difficult, especially, with conifers. Mature tissues of *Pinus sylvestris* L. have a very low morphogenic potential *in vitro* (Bonga 1987; Hohtola 1988).

Several methods of pretreatment for the rejuvenation of mature conifer tissues or the increase of their morphogenic potential have been described – serial grafting (Huang et al. 1992; Ewald, Kretzschmar 1996; Centeno et al. 1998), cold or frozen storage of detached resting buds (Andersone, Ievinsh 2002), spraying of donor plants with cytokinin (Salonen 1991). The timing of collections is also a critical factor (Bonga 1987; Andersone, Ievinsh 2002). However, only brachioblast and needle formation is achieved on mature Scots pine bud explants (Andersone, Ievinsh 2002). Cultivation of the explants on media containing cytokinin did not result in new bud or shoot formation. One of the reasons for that could be very low cytokinin response-competence of the mature *P. sylvestris* tissue.

It is well known that bud production or adventitious branching is usually achieved by increasing the cytokinin level in the tissues. However, extremely rapid multiplication

in tissue culture due to high levels of cytokinin may lead to genetic changes (Peschke, Phillips 1992). It is also known that a high concentration of cytokinin, while stimulating organ induction, inhits their elongation and development (Salonen 1991). Therefore, pulse treatment with cytokinin between subcultures can be used to achieve a high level of multiplication without undesirable genetic effects. In experiments with mature intact conifer trees, repetitive cytokinin treatment has been used for juvenilization leading to development of new shoots (Krikorian 1982).

The aim of the present study was to investigate the possibility of using a pulse treatment with cytokinin as well as to affect cytokinin response competence in mature P. *sylvestris* tissues by means of cold treatment of established *in vitro* culture of bud explants.

Materials and methods

Plant material was collected from mature pine (*Pinus sylvestris* L.) trees in a seed orchard near Salaspils (Riga Region, Latvia). Buds were taken randomly from different trees from the lower part of the crown. Collection was carried out from the second half of July till the first half of August. Newly formed buds were used. They were surface sterilized with a half-diluted commercial bleach ACE (Procture and Gamble; containing 5 - 15 % sodium hypochlorite) for 20 min, rinsed for 10 min in sterile distilled water, sterilized again in 15 % hydrogen peroxide and rinsed three times for 10 min in sterile distilled water. The buds were peeled and dissected aseptically. Explants were cultivated in 20 × 200 mm glass test-tubes containing 10 ml agarized nutrient medium. Tubes were closed with cotton-wool

Table 1. Media used for cultivation of *Pinus sylvestris* bud explants

	Basal medium (BM)	Medium No. 1 (M1)	Medium No. 2 (M2)	Medium No. 3 (M3)
Woody Plant Medium mineral salts	+	+	+	+
Myo-inositol	100 mg l^{-1}	100 mg l^{-1}	100 mg l^{-1}	100 mg l^{-1}
Thiamine hydrochloride	30 mg l^{-1}	30 mg l^{-1}	30 mg l^{-1}	30 mg l^{-1}
Pyridoxine hydrochloride	10 mg l^{-1}	10 mg l^{-1}	10 mg l^{-1}	10 mg l^{-1}
Nicotinic acid	10 mg l^{-1}	10 mg l^{-1}	10 mg l^{-1}	10 mg l^{-1}
Glycine	1 mg l^{-1}	1 mg l^{-1}	1 mg l^{-1}	1 mg l^{-1}
Benzylaminopurine	–	–	100 mg l^{-1}	–
Naphthylacetic acid	–	0.1 mg l^{-1}	0.2 mg l^{-1}	0.2 mg l^{-1}
Adenin	–	10 mg l^{-1}	–	250 mg l^{-1}
Kinetin	–	1 mg l^{-1}	–	25 mg l^{-1}
Sucrose	45 g l^{-1}	45 g l^{-1}	45 g l^{-1}	45 g l^{-1}
Agar	7 g l^{-1}	7 g l^{-1}	7 g l^{-1}	7 g l^{-1}
pH	5.6 - 5.7	5.6 - 5.7	5.6 - 5.7	5.6 - 5.7

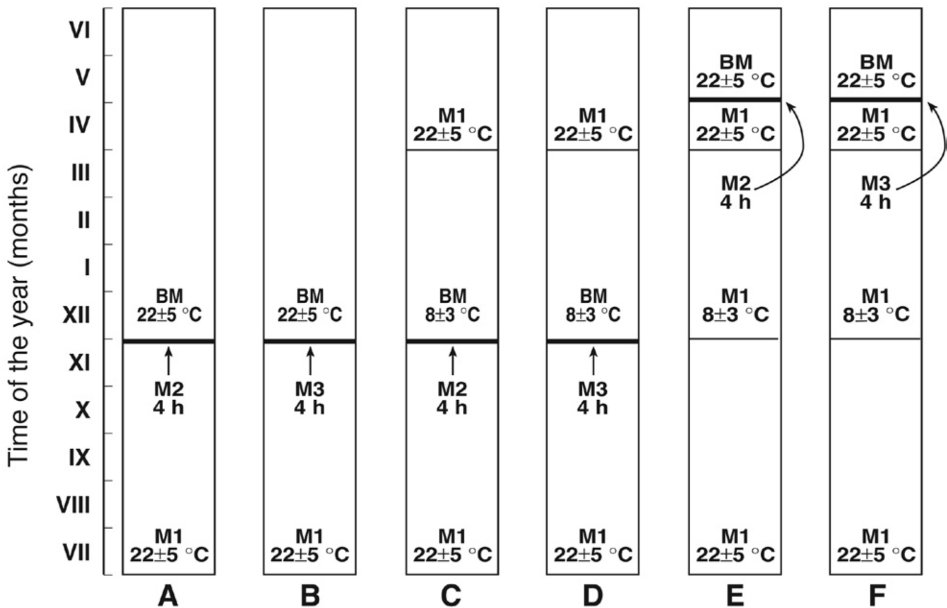

Fig. 1. A diagram outlining the experimental procedure. Time sequence, cultivation media used, as well as temperature regimes are shown for experimental variants A to F.

plugs, and covered also with polythene film fixed with a rubber band. Each tube contained one explant. During the entire time they were cultivated in natural lighting.

For culture establishment medium M1 was used (Table 1). Pulse treatment with high concentrations of cytokinins was carried out on media M2 (containing benzylaminopurine, BAP) or M3 (containing kinetin and adenin) for 4 h. After the cytokinin treatment, basal medium (BM) without growth regulators was used. During cold storage, explants were cultivated at 8 ± 3 °C; control explants were maintained at 22 ± 5 °C.

Six different variants of cytokinin treatment in combination with or without cold storage were tested (Fig. 1). For each variant three replicates of 10 bud explants per treatment were used.

Results

Pulse treatment with cytokinins of pine bud explants cultivated at 22 °C (variants A and B) did not result in increased morphogenesis (Table 2). New bud and shoot formation was achieved on pine bud explants (variants C and E) which were pulse-treated with BAP in combination with cold incubation (Table 2). The effect was more pronounced when the treatment was performed after the cold incubation (40 %) in comparison with the treatment performed before the cold incubation (20 %). Also, the average amount of microshoots per one shoot-forming explant was higher in variant E than in variant C (Table 2). Kinetin in combination with adenin did not result in bud and shoot formation (Table 2, variants D and F).

In both variants of BAP treatment in combination with cold incubation (variants C

Table 2. The effect of cold storage on cytokinin response-competence of mature *Pinus sylvestris* tissues *in vitro*. Data are means from 3 replicates (10 explants per treatment) ±SE

	Treatment					
	A BAP	B Kinetin + adenin	C BAP followed by cold	D Kinetin + adenin followed by cold	E Cold followed by BAP	F Cold followed by kinetin + adenin
Bud explants, forming microshoots (%)	0	0	20 ± 2	0	40 ± 3	0
Microshoots per explant	0	0	5.5 ± 0.8	0	6.8 ± 1.2	0

and E), buds and microshoots developed directly on the lower part of the explants (Fig. 2A, D). Elongation of these buds occurred only in variant E (Fig. 2D). In variants C and E, also brachioblasts with short needles were formed on upper and middle part of explants (Fig. 2A, D). In variant C, axillary shoots developed between these needles and subsequentially elongated (Fig. 2B). The longest of these elongated axillary microshoots were isolated and transferred to a fresh BM medium (Fig. 2C).

Discussion

In previous experiments with *P. sylvestris* it was found that about 15 % of mature pine buds introduced *in vitro* in the second half of summer form brachioblasts with needles (Andersone, Ievinsh 2002). However, further development of these explants was not obtained: buds with needles as well as buds without needles did not survive longer than eight months *in vitro*. No further multiplication was achieved even on media with high concentrations of cytokinins. This situation is similar to the observation that micropropagated plants of mature origin may retain their physiological maturity during tissue culture (Nas et al. 2003). Consequently, inability to respond to growth regulators, e.g. cytokinins, is one of the characteristics of the "out of hormonal response competence" state of mature tissues during *in vitro* cultivation.

It has been suggested that accumulation of competence to induction is important before a particular induction phase during shoot organogenesis (Christianson, Warnick 1988). Thus, to deal with a lack of induction competence in mature pine explants reflected in lack of cytokinin-response competence, certain conditions should be met. As a possible candidate for such conditions, cold treatment can be used, as it was shown for intact juvenile *P. sylvestris* that cold exposure is necessary for elongation of cytokinin treatment-induced buds (Salonen 1991). In order to improve cytokinin response-competence of pine bud tissues, cold treatment of established *in vitro* cultures at 8 °C was used in the present experiments.

Due to a relatively slow transport of exogenous cytokinins, the concentration of BAP in pine bud tissues obviously decreased in the direction towards the apical part of the bud, explaining why the largest amount of shoots formed at the lower part of the explants. In

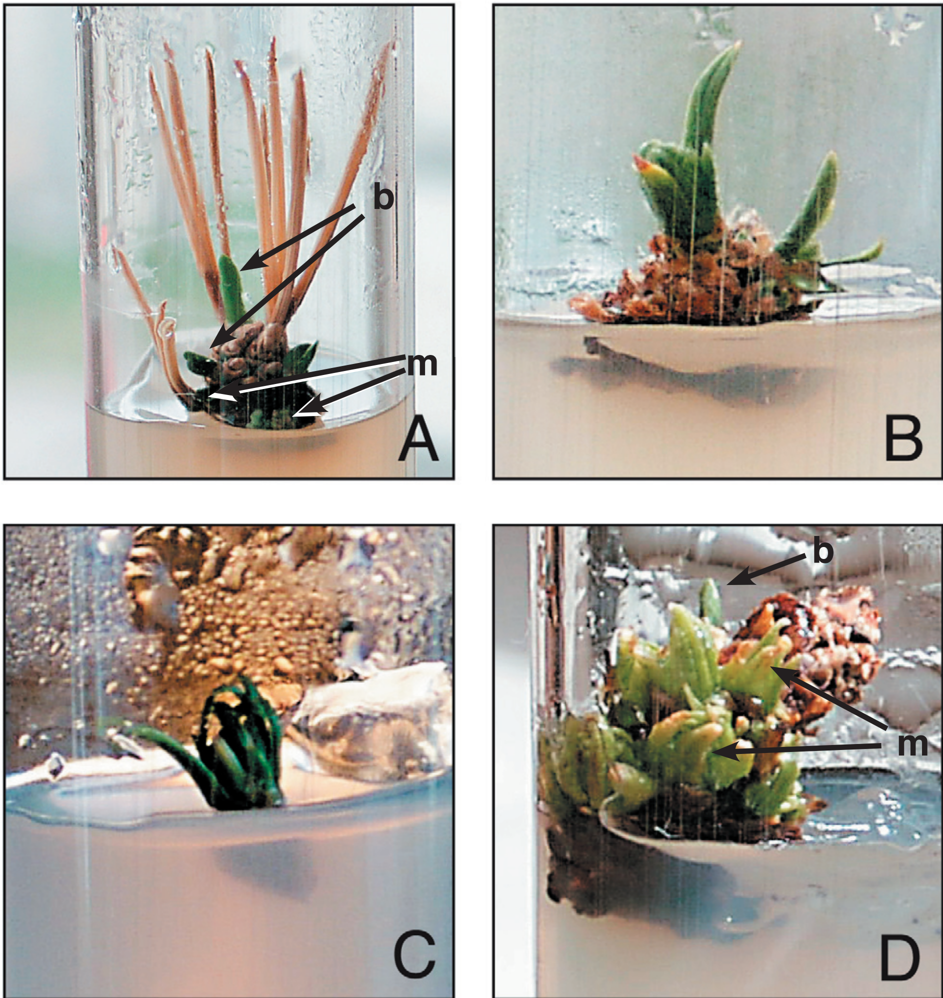

Fig. 2. Development of mature *Pinus sylvestris* bud explants *in vitro* under the effect of cold and cytokinin treatment. A, experimental variant C (according to Fig. 1, pulse treatment with BAP before cold incubation), 4 weeks after the end of the cold incubation; B, variant C, 6 weeks after the end of the cold treatment; C, variant C, isolated axillary shoot on BM medium, 2 weeks after isolation; D, variant E (pulse treatment with BAP after cold incubation), 6 weeks after the BAP treatment. m, microshoots; b, brachioblast with needles.

contrast, secondary needles were formed only in the central or upper part of the pine bud explants, which is in accordance to the observation that a high concentration of cytokinin prevents secondary needle formation (Zhang et al. 2003). Our results were similar to those obtained with *Actinidia deliciosa* explants, where gradient of exogenously applied BAP was responsible for different developmental patterns (Feito et al. 2001).

The effect of cytokinin was more pronounced when the pulse treatment with BAP was performed after the cold incubation (variant E), in comparison with the pulse treatment

before the cold incubation (variant C). Consequently, in the latter variant, during the development of competence in conditions of cold treatment, exogenously applied BAP was considerably decomposed in pine tissues leading to less pronounced cytokinin response. Decomposition of exogenously applied citokinin-like substances in plant tissues is a well known phenomenon (Harrison, Kaufman 1984).

In conclusion, due to the temporary nature of juvenilization of mature pine bud explants, it is necessary to combine both physical and biochemical means to promote morphogenic competence and to achieve further development in tissue culture.

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