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Medium pH affects regeneration capacity and oxidative enzyme activity of *Pinus sylvestris* in tissue culture

Una Andersone*, Gederts Ievinsh

Department of Plant Physiology, Faculty of Biology, University of Latvia, Kronvalda Bulv. 4, Rīga LV-1586, Latvia

*Corresponding author, E-mail: una.andersone@inbox.lv

Abstract

Medium pH that is one of the important factors of the physico-chemical environment during development of plant tissues in conditions of *in vitro* culture which is modified during preparation of media, but also changes with developing explants. The present experiments were performed to monitor the changes of medium pH and to determine the effect of medium pH on physiological characteristics of mature *Pinus sylvestris* L. tissue cultivated *in vitro*. The final changes of medium pH after addition of agar and autoclaving were towards alkalinity in the range of initial pH 3.0 to 5.5. Results of the experiments proved that in general cultivation of pine buds causes acidification of the medium. The degree of acidification of medium during cultivation depends on initial pH of medium, morphogenesis of explants and duration of cultivation period without transplantation. Lowered activity of oxidative enzymes in buds cultivated on more acidic medium and less necessity for acidification to reach the equilibrium in medium pH suggest that a low initial pH is more suitable for successful pine bud morphogenesis.

Key words: medium pH, morphogenesis, peroxidase, *Pinus sylvestris*, polyphenol oxidase, Scots pine, tissue culture.

Introduction

Medium pH is one of the important factors of physico-chemical environment during development of plant tissues in conditions of *in vitro* culture (Williams et al. 1990). The optimal pH of *in vitro* medium for different phases of morphogenesis (successful *in vitro* culture establishment, intensive shoot proliferation, induction of the rooting) for woody species varies (von Arnold, Eriksson 1982; Williams et al. 1985; Saborio et al. 1997; Ostrolucka et al. 2004).

Suboptimal pH levels can cause abnormalities in the development of explants e.a. reduction in growth of the hypocotyls, stem and roots, changes in leaf coloring (Gurel, Gulsen 1998; Laukkanen et al. 2000). Optimal pH of media *in vitro* can be different from that of soil *ex vitro* (Sen et al. 1994) and in *ex vitro* acclimatization in hydroponic culture (Ingestad 1979; Bozhkov, von Arnold 1998).

In tissue culture practice pH of medium is adjusted before autoclaving and changes of pH during autoclaving and cultivation of tissues are usually ignored. The changes during autoclaving depend upon the initial pH and characteristics of the gelling agent (Williams et al. 1990; Van Winkle, Pullman 2003). Contrary to common belief, the changes of medium

pH during cultivation are not a response to wounding. Although wounding stimulates the decrease in pH over the first few days, the effect is insignificant (Williams et al. 1990).

Plant tissues can maintain a relatively constant cytoplasmic pH across an external pH range of 4 to 9 (Caponetti et al. 1971). Plant cells can also modify the external pH – the explants raise or lower the pH, depending on which pH range they grow, until an equilibrium occurs (Mac AntSaoir, Damvoglou 1994). A localized change of pH takes place at the site of contact between the plant tissue and the medium in tissue culture as well as in soil (Constable 1963; Haussling et al. 1985).

For tissue culture of different *Pinus* species pH 5.5 to 6.0 is used (Durzan, Chalupa 1976; Sen et al. 1994; Saborio et al. 1997; Sul, Korban 2004; Tang et al. 2004). *Pinus sylvestris* L. usually is cultivated in media with pH 5.5 to 5.8 (Bornman, Jansson 1980; Hohtola 1988; Žel et al. 1988; Supriyanto, Rohr 1994; Laukkanen et al. 1997; Laukkanen at al. 1999; Lelu et al. 1999; Laukkanen et al. 2000). Conifers can also change the media pH during cultivation. Embryogenic cultures of Norway spruce, loblolly pine and Douglas-fir tend to decrease the pH of growth medium (Van Winkle, Pullman 2003), but suspension culture of *Pinus banksiana* slightly increases pH of the medium during cultivation (Durzan, Chalupa 1976).

The present experiments were performed to study changes of medium pH and the effect of medium pH on physiological characteristics of mature *Pinus sylvestris* tissue cultivated *in vitro*. Oxidative enzyme activities (peroxidase and polyphenol oxidase) were used as indicators of morphogenic potential (Andersone, Ievinsh 2005).

Materials and methods

Plant material

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. Buds were surface sterilized with a half-diluted commercial bleach ACE (Procture and Gamble; containing 5 to 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.

Culture conditions and media

Explants were cultivated in 40 × 100 mm glass tubes containing 20 mL agarized medium (for experiments with medium pH measurements after autoclaving) or in 20 × 200 mm glass test-tubes containing 10 ml agarized nutrient medium (for experiments with medium pH measurements before adding agar and autoclaving). Vessels were closed with cotton-wool plugs, and covered with polythene film fixed with a rubber band. Each vessel contained one explant. They were cultivated at temperature 20 ± 5 °C in natural light (if not indicated otherwise).

Explants were cultivated on Woody Plant Medium (Lloyd, McCown 1981) as modified by Andersone and Ievinsh (2002). 0.5 mM naphtyl acetic acid, 54 mM adenine, and 4.7mM kinetin were used as growth regulators. The medium was supplemented with 0.6 mM myo-inositol, 88.9 mM thiamine hydrochloride, 48.6 mM pyridoxine hydrochloride, 81.2 mM nicotinic acid and 131.4 mM sucrose. It contained 0.57 % of plant agar.

For experiments on the effect of tissues on medium pH, medium was adjusted to 5.8 before adding agar and autoclaving. For experiments of effect of medium pH on tissue development, pH was adjusted 3.0 to 7.0 with interval pH 0.5 before adding agar and autoclaving. The adjustment was performed either by 1N HCl or 1N KOH.

Measurement of pH

Measurements of pH were carried out with a pH meter pH 211, by immersion of the electrode HI 1131B (Hanna Instruments). For measurement of medium pH before autoclaving the electrode was stirred in the liquid medium before agar was added. For measurement of media after autoclaving the electrode was imbedded in it two days after autoclaving. To investigate medium pH changes after cultivation of mature tissue, pH was measured by placing the electrode into the hole from which the bud explant was removed.

Effect of media preparation

To determine changes of pH during media preparation the appropriate mineral salts and organic components of the medium were mixed and pH was adjusted (2.5 to 7.0 with 0.5 intervals). Agar was then added, dissolved in a half of the volume by heating, media were dispensed into cultivation vessels and autoclaved at 121 °C and 103 KPa for 20 min. The pH in each vessel was measured two days later.

Effect of in vitro *cultivated bud tissue on pH of the medium*

Buds were cultivated *in vitro* and placed on fresh medium (pH 5.8) weekly or after every 2, 3, 4 or 5 weeks. The third part of the explants were cultivated in a growth chamber with a 16 h photoperiod, where illumination was provided by fluorescent tubes OSRAM L 36/W77, the rest were cultivated near a window in laboratory under natural light with a brief period of direct sunlight or with a prolonged period of direct sunlight. Twenty sterile explants were taken for each variant. pH of the media was measured after autoclaving and after removal of bud explants weekly or after every 2, 3, 4 or 5 weeks. The changes of pH during cultivation were estimated for 6 to 8 weeks. The experiment was carried out for 10 months. The morphological condition of bud explants (development of brachyoblasts and needles, necroses) was recorded during cultivation and at the end of experiment.

Effect of medium on in vitro *cultivated bud tissue*

Buds were cultivated on media with different pH levels (pH 3.0 to 7.0 with intervals pH 0.5) adjusted before autoclaving.

To investigate the changes of medium pH during cultivation, buds were cultivated for two months, then transplanted on fresh medium with the same variant of pH and cultivated for 10 months. Media pH was measured after removal of cultured buds. To investigate the effect of different medium pH on bud morphogenesis, morphological characteristics (needle formation, amount of needles, length of needles, necroses) were recorded after 2, 4 and 8 months of cultivation.

To investigate the effect of different medium pH on activity of peroxidase and polyphenol oxidase, buds, cultivated in vitro for seven weeks, were frozen in liquid nitrogen and ground to fine powder with a mortar and pestle. The experiment was repeated twice. Enzymes were extracted with 25 mM HEPES/KOH buffer (pH 7.2) containing 1 mM

EDTA, 3 % (w/v) PVPP and 0.8 % (v/v) Triton X-100 for 15 min at 4 °C. The homogenate was centrifuged at 15 000 g for 20 min. The supernatant was used for assays.

Peroxidase activity was measured spectrophotometrically at 470 nm in reaction mixture containing 2 mL 50 mM sodium phosphate buffer (pH 7.0) with 10 mM guaiacol, 0.5 mL 0.03 M H_2O_2 and 0.01 mL enzymatic extract. The reaction mixture without H_2O_2 was used as a reference.

The activity of polyphenol oxidase was determined spectrophotometrically in a reaction mixture (3 ml) containing 20 mM sodium phosphate (pH 6.5) with 25 mM pyrocatechol and the enzymatic extract (0.01 mL). The change in absorbance was monitored at 410 nm.

Results

During medium preparation pH changed towards alkalinity. Strongly acidic (pH 2.5) or alkaline (6.0 to 7.0) media did not change significantly during autoclaving (Fig. 1). Results of both experiments proved that in general, cultivation of pine buds caused acidification of the medium (Fig. 1 to 4).

During bud cultivation on media with different initial pH the final pH was similar. Two- and 10-month long cultivation of buds on media with pH 3.5 to 7.0 resulted in almost the same acidity of medium for all variants of pH. After 2 months of cultivation the mean pH of medium was 5.26 ± 0.06, but in 10 months cultivation the mean medium pH declined to 4.18 ± 0.11 (Fig. 1). Buds, cultivated on medium with alkaline initial pH, acidified the medium more than buds cultivated on medium with initially acidic pH.

The rate of acidification depended on the duration of cultivation without transplantation. The maximal rate of acidification was observed during the first four weeks of cultivation,

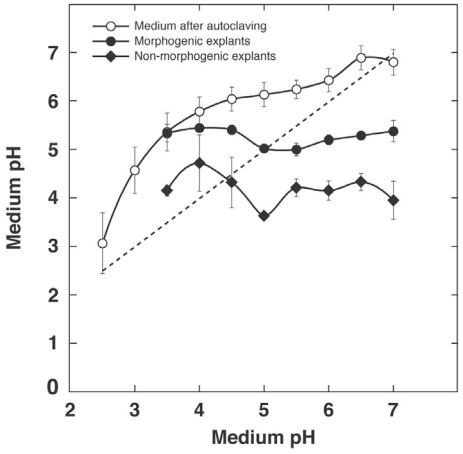

Fig. 1. Change of medium pH after autoclaving and with different types of pine bud explants during 10 months of cultivation without transplanting. Dashed line indicates no change in the pH values.

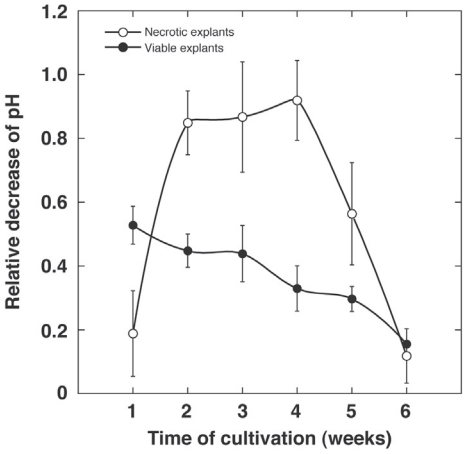

Fig. 2. Effect of time of cultivation and explant type on relative decrease of medium pH. Initial pH of the cultivation medium was 5.8 (6.25 after autoclaving). Explants were transplanted to the fresh medium with initial pH value every week.

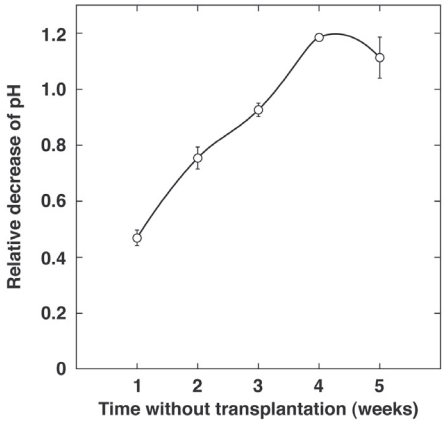

Fig. 3. Effect of time of cultivation before transplanting on relative decrease of medium pH. Initial pH of the cultivation medium was 5.8 (6.25 after autoclaving). Explants were transplanted on a fresh medium with initial pH after the indicated intervals of time and cultivated for 10 months.

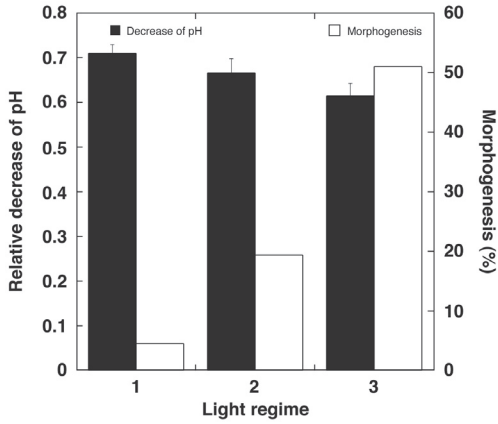

Fig. 4. Effect of illumination conditions on relative decrease of medium pH and percentage of morphogenesis in pine bud explants. Artificial light only (1), natural light with short periods of direct sunlight (2), natural light with long periods of direct sunlight (3). Initial pH of the cultivation medium was 5.8 (6.25 after autoclaving). Explants were cultivated for 10 months without transplanting.

then decreased, but slower acidification continued when explants were not transplanted for 10 months (Fig. 1, 3). In the first cultivation week acidification was from pH 6.25 to 5.76 (acidification rate 0.49), but in 10 months time from pH 6.25 to 4.18 (acidification rate 0.048 weekly).

The results of our experiments showed that the degree of acidification depended on the physiological condition of explants. To evaluate if morphogenesis affected the ability of pine buds to change medium pH buds were divided into groups according to different developmental events or lack of development. If constructive processes in buds dominated over destructive, medium acidification was less marked than for buds with dominating destructive processes. This relationship was characteristic for buds in the first experiment, transplanted every week (Fig. 2), for buds transplanted every 2, 3, 4 or 5 weeks (data not shown) or grown for 10 months without transplantation in the second experiment, when average medium pH with needle forming buds was 4.59 ± 0.15, but for buds without needles it was 3.91 ± 0.08.

The same relationship was characteristic also for buds, cultivated in different light intensities. Change of medium pH for different light intensities in the first experiment was lowest for explants cultivated in natural light with a prolonged period of direct sunlight (greater percentage of viable buds with enlarged brachioblasts and needle-forming buds), and highest for explants cultivated in artificial light that did not form needles and became necrotic sooner (Fig. 4).

The second experiment was performed to test if the initial pH of the medium affects pine tissue development. The morphological characteristics of buds (average percentage of needle forming buds, average amount of needle pairs on each needle forming bud, length of needles) varied (Fig. 5, 6A), but on the whole the results were not convincingly

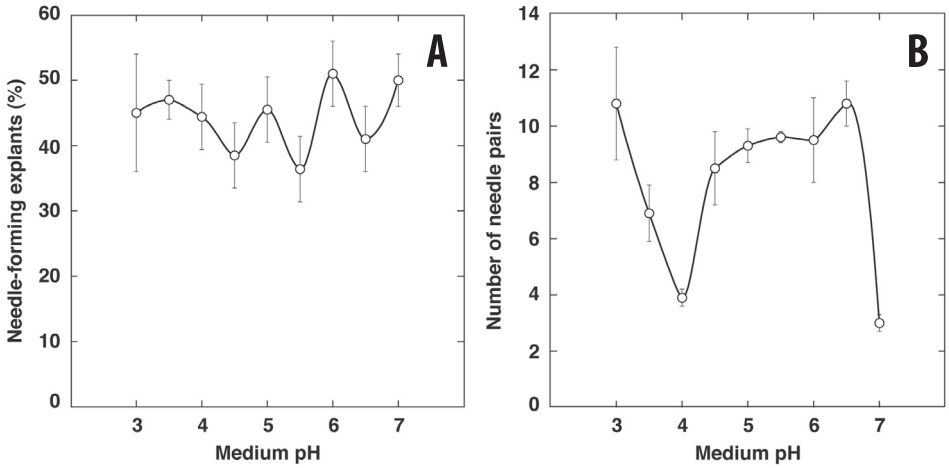

Fig. 5. Effect of initial medium pH on percentage of needle-forming explants (A) and number of needle pairs per needle-forming explant (B) within 10 months of cultivation. Explants were cultivated without transplanting.

affected by initial pH of medium.

The percentage of necrotic tissue in buds cultivated *in vitro* for four months (second experiment) was lowest on media with the most acidic and most alkaline initial pH (Fig. 6B).

The activity of peroxidase in buds cultivated *in vitro* for 7 weeks on media with different pH was significantly lower for buds cultivated on pH 3.0, increased for higher pH values up to 5.0, but lowered again and stopped decreasing at pH 6.5 (Fig. 7A). The activity of polyphenol oxidase in the same buds was also lowest for pH 3.0 and increased up to pH 6.5 (Fig. 7B).

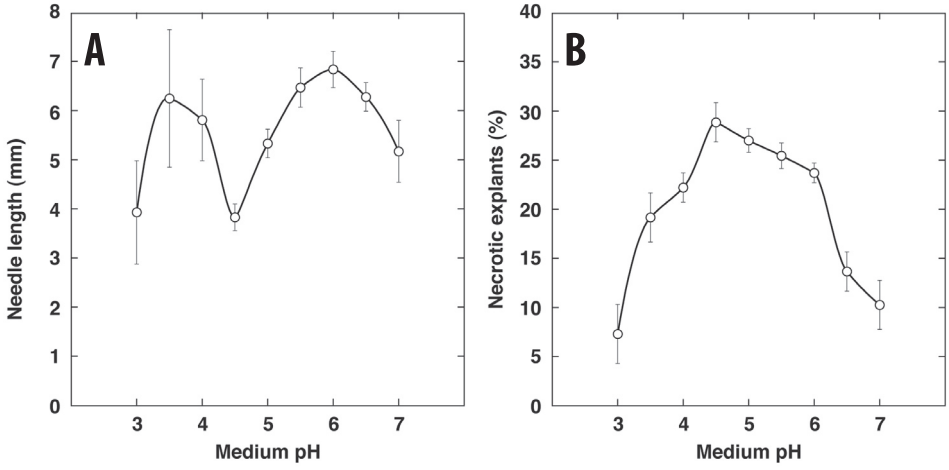

Fig. 6. Effect of initial medium pH on percentage of needle length (A) and percentage of necrotic pine bud explants (B) within 10 months of cultivation. Explants were cultivated without transplanting.

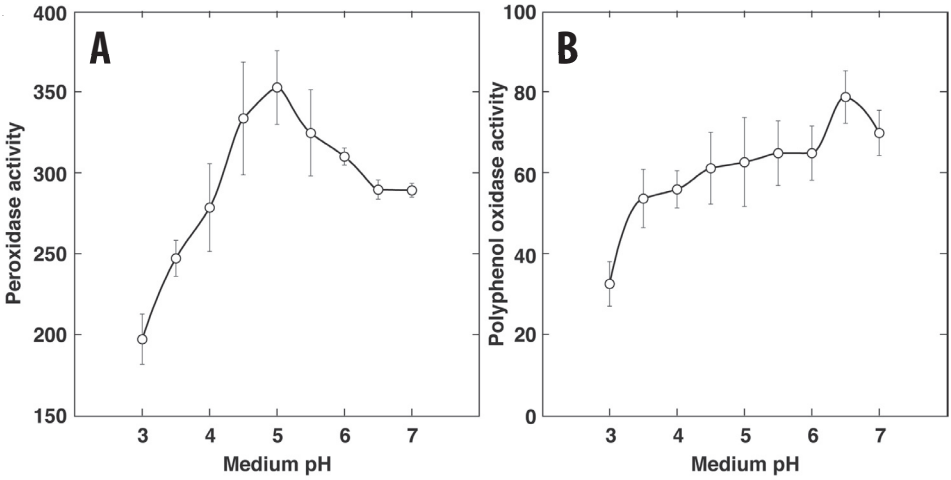

Fig. 7. Effect of initial medium pH on peroxidase (A) and polyphenol oxidase (B) activity in pine bud explants after 7 weeks of cultivation. Explants were cultivated without transplanting.

Discussion

Changes of medium pH should be taken in to the account when using plant tissue culture both for practical purposes as well as for research. Changes of pH can occur from preparation of medium until *ex vitro* transplantation and are affected both by interactions between chemical constituents as well as plant tissues and medium constituents.

Adjustment of medium pH is usually performed before adding agar, but the agar itself and subsequent autoclaving change medium pH. The addition of agar to the nutrient solution raised the pH when initial pH was below 5.5, but autoclaving decreased pH, except when pH was lower than 4.0 (Williams at al. 1990). In our investigation we recorded only the final medium pH after addition of agar and autoclaving which were towards alkalinity (Fig. 1). Differences in pH may arise from different types of agar, other medium components or medium preparation features.

Medium pH changed during cultivation of tissues usually towards acidity. The degree of acidification of the medium during cultivation is influenced by several factors, including the source of nitrogen, physiological status of cultivated tissues, duration of cultivation, frequency of transplanting etc.

The source of nitrogen in the medium and its utilization by cells can play an important role in determining the shift in the pH value of the medium (Sathyanarayana, Blake 1994). Plant growth *in vitro* can occur regardless of initial pH as the medium contains both N forms (Mac AntSaoir 1994). Nitrogen uptake as nitrate is higher at an acidic pH and shifts the medium pH towards alkalinity, while ammonium utilization rate increases with increasing pH and shifts the medium pH towards acidity (Gamborg, Shyluk 1970; Martin, Rose 1976; Wetherell, Dougall 1976; Dougall, Verma 1978). In *Pinus sylvestris* callus cultures the medium became acidic when it contained NH_4NO_3 (from pH 5.7 to 4.3), but the pH of the medium containing KNO_3 remained close to the initial value (from pH 5.7 to 5.4; Laukkanen et al. 1997). Seedlings of a number of forest tree species, including *Pinus sylvestris* grow better when nitrogen is supplied as ammonium or as ammonium

plus nitrate, and when ammonium is preferred as the nitrogen source, the rhizosphere will be acidified by induced H^+ extrusion (Arnold 1992). In our second experiment where medium contained ammonium and nitrate pine bud explants changed the medium pH to a constant value for the whole range of initial pH of medium (Fig. 1).

The degree of acidification in our experiments depended on the morphogenesis of explants. Needle formation on buds was associated with less acidified medium. From the results of the experiment on light intensity and changes of medium pH we can also conclude that pH change was less for natural illumination conditions, which stimulated morphogenesis, and promoted enlarged brachyoblast development and needle formation (Fig. 4). The degree of acidification of medium during cultivation depended also on the duration of cultivation period before transplantation. The observed change of medium pH for different cultivation periods before transplantation shows that it is not necessary to transplant pine buds to fresh medium more often than every two months or even less frequently.

The initial pH of medium also affects the degree of acidification during cultivation. If the initial pH is more alkaline, explants consume more energy to acidify the medium than explants cultivated on medium with initially acidic pH. Moreover production of acidic compounds by the mycobacterium living in pine buds may also cause acidification of the growth medium (Laukkanen et al. 2000).

Medium pH was changing due to release of some compounds from the plant or as a result of uptake of specific ions from the medium. Tissue secretes phytosiderophores, organic acids and proteins, which may react with metal ions present and change chemical dissociation kinetics, in turn altering their bioavailability (Friborg et al. 1978; Egertsdotter et al. 1993; Zhang 1993; Van Winkle, Pullman 2003). It is possible that buds becoming necrotic secrete into the medium compounds different from those of needle forming buds, which may result in different effects on medium pH.

Although the difference in initial pH may have a significant effect on the culture system even though the pH difference is eliminated after some time of cultivation (Williams et al 1990), the obtained morphological data in our experiment do not show any simple trend of relationship between initial pH of medium and development of explants (Fig. 5, 6). In the acidic part of medium pH range the morphological development occurred during longer cultivation periods and the morphological parameters were better and buds less necrotic. For *Pinus sylvestris* callus cultures a lower pH in the presence of ammonium in culture medium correlated with better growth (Laukkanen et al. 1997). However, differences between pH variants may be caused also by biological diversity of the material used for experiments.

In our study peroxidase and polyphenol oxidase activity was lowest in the acidic end of medium pH range (Fig. 7). A low peroxidase and polyphenol oxidase activity correlated with increased morphogenic potential of mature *Pinus sylvestris* buds *in vitro* (Andersone, Ievinsh 2002; Andersone, Ievinsh 2005). Buds cultivated on initially more acidic medium need less acidification to reach equilibrium. These results may confirm the morphological results of the experiment with different initial pH of medium, e.g. that mature *Pinus sylvestris* buds *in vitro* need acidic initial medium pH (pH 3.0 to 3.5 before adding agar and autoclaving). The presence of ammonium in culture medium might have an effect on polyphenol oxidase and peroxidase by decreasing the pH and altering the water potential of the cells. However, increased browning in the presence of only nitrate

may result in significantly higher pH, because at higher pH levels phenolics will oxidize to form quinones, which will bind irreversibly to proteins in a non-enzymatic fashion (Laukkanen et al. 1997).

In conclusion the degree of acidification of medium during cultivation of mature *Pinus sylvestris* buds depends not only on the source of nitrogen, products of mycobacteria living in pine buds as described previously, but also on initial pH of medium, morphogenesis of explants and duration of cultivation period without transplantation. Lowered activity of oxidative enzymes in buds cultivated on more acidic medium, and that needed less acidification to reach equilibrium, suggests that low initial pH is more suitable for successful pine bud morphogenesis.

Acknowledgements

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Barotnes pH ietekmē reģenerācijas spējas un oksidatīvo fermentu aktivitāti *Pinus sylvestris* audu kultūrā

Una Andersone*, Ģederts Ieviņš

Augu fizioloģijas katedra, Latvijas Universitātes Bioloģijas fakultāte, Kronvalda bulv. 4, Rīga LV-1586, Latvija
*Korespondējošais autors, E-pasts: una.andersone@inbox.lv

Kopsavilkums

Barotnes pH ir viens no svarīgākajiem fizikālķīmiskās vides faktoriem augu audu attīstības laikā *in vitro* kultūras apstākļos. To ietekmē barotnes sagatavošana un tas mainās arī līdz ar eksplantu attīstību. Dotos ekesperimentus veica, lai novērotu barotnes pH izmaiņas un noteiktu barotnes pH ietekmi uz *in vitro* kultivētu nobriedušu *Pinus sylvestris* L. audu fizioloģiskajām īpašībām. Pēc agara pievienošanas un autoklavēšanas barotnes ar sākuma pH no 3.0 līdz 5.5 kļuva sārmainākas. Pētījumu rezultāti apstiprināja, ka priežu pumpuru kultivēšana izraisa barotnes paskābināšanos. Barotnes paskābināšanās pakāpe bija atkarīga no sākotnējā barotnes pH, eksplantu morfoģenēzes un kultivēšanas ilguma bez pārstādīšanas. Pazemināta oksidatīvo fermentu aktivitāte pumpuros uz skābākas barotnes un īsāks šādu kultūru pH stabilizācijai nepieciešamais laika periods liecina, ka veiksmīgai priežu pumpuru morfoģenēzei nepieciešams zemāks sākotnējais barotnes pH.