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Growth regulators and guaiacol peroxidase activity during the induction phase of somatic embryogenesis in *Picea* species

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Abstract: Biochemical studies during the induction phase of somatic embryogenesis in *Picea abies* [L.] Karst. and *P. omorika* [Pančić] Purk. can supplement our basic knowledge of the developmental processes accompanying the formation of embryogenic tissues from explants. Such studies may also contribute to finding the markers specific to the early stages of somatic embryogenesis of spruce species and, consequently, to the optimization of the process of initiation of embryogenic tissues from different types of plant explants treated with various growth regulator combinations.
In this paper the effect of certain growth regulator systems on enzymatic activity was studied. The analysis of guaiacol peroxidase activity (EC 1.11.1.7), based on the spectrophotometric method, showed that this activity was the lowest in mature zygotic embryos (explants) and significantly higher in 8-week-old embryogenic and non-embryogenic calluses treated with various combinations of growth regulators. In the newly initiated embryogenic tissue, the activity of this enzyme decreased and remained at a lower level during proliferation, irrespective of the applied growth regulator combination. The type and concentration of growth regulators used for the initiation and proliferation of embryogenic tissues had no statistically significant effect on peroxidase activity, although during the initiation often its increased level was observed in calluses treated with 2.4-D.
Detection of guaiacol peroxidase activity in the induction phase of somatic embryogenesis proves its participation in this process. The subsequent change in its activity indicates that this peroxidase can be a biochemical marker of somatic embryogenesis of the tested spruce species.

Additional key words: spruce, embryogenic tissue, auxins, peroxidase

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Introduction

Somatic embryogenesis is the process of somatic cells acquiring embryogenic competence under specific conditions. These cells undergo a series of morphological, physiological, molecular and biochemical changes that lead to the formation of somatic embryos (Quiroz-Figueroa et al. 2006). The process of somatic embryogenesis can be controlled and regulated in vitro.

Embryogenic tissue of coniferous tree species is usually initiated from immature or mature zygotic embryos taken from seeds (Vágner et al. 2005; Pullman et al. 2009). Explants are placed in media con-

taining growth regulators (i.e., plant growth regulators), which are usually auxins and cytokinins (Vágner et al. 2005), or as in the case of firs, cytokinins alone (Salajova et al. 1998), at a suitable concentration to initiate somatic embryogenesis. The type and physiological status of explants and the type and concentration of growth regulators are among the main factors determining the acquisition of embryogenic competence by plant cells in vitro, although genetic factors also affect this process (Namasivayam 2007).

Somatic embryogenesis can be divided into induction and expression phases. In the induction phase, the somatic cells of explants acquire embryogenic competence (initiation stage) and proliferate as embryogenic cells (proliferation stage). In the expression phase, the embryogenic cells differentiate into somatic embryos (Namasivayam 2007). Jimenez (2001) suggests that these two phases (induction and expression) are independent of each other and influenced by different factors.

The acquisition of embryogenic competence by vegetative cells and the further development of somatic embryos are associated with many biochemical changes, which often result from the influence of plant growth regulators added to the medium at various stages of the culture. These changes are reflected e.g. in differences in the activity of specific enzymes closely related to the different phases of somatic embryo development: from the initiation of embryogenic tissue (ET) from explants to the formation of complete plants (Mo et al. 1996; Kormuták et al. 2003; Quiroz-Figueroa et al. 2006). Some of the enzymes which activity often changes during somatic embryogenesis in plants are peroxidases (Krsnik-Rasol 1991; de Klerk 1997; Stasolla and Yeung 2007). Peroxidases (EC 1.11.1.7) belong to the group of oxidoreductases, catalyzing hydrogen peroxide oxidation of various substrates. In plant cells, peroxidase isoenzymes are located in the cell wall and vacuole (Carpin et al. 1999). They participate in the antioxidant system, protecting cells from excessive accumulation of hydrogen peroxide and pathogen attack (Sutherland 1991) as well as in plant cell development (Brownleader et al. 2000; Cordewener et al. 1991), e.g. in somatic embryos of coniferous tree species (Mo et al. 1996; Kormuták et al. 2003).

The aim of this study was to determine whether guaiacol peroxidase is active during the induction phase of somatic embryogenesis in *P. abies* [L.] Karst. and *P. omorika* [Pančić] Purk. and if the type or concentration of growth regulator combinations affects the level of its activity and the level of ET initiation and proliferation.

Material and methods

ET initiation and peroxidase activity

Cones of *Picea abies* and *P. omorika* were collected from the local experimental forest *Zwierzyniec* (provenance Kolonowskie) and from the Arboretum in Kórnik near Poznań (Poland) in October 2009, respectively. The seeds were stored at 4°C for 2–3 months. After this time, the seeds were sterilized for 10 minutes in 33% (v/v) H_2O_2 (hydrogen peroxide solution) with addition of two drops of Tween 20, rinsed three times in sterile distilled water and stored in fresh water for 14–16 hours in the dark at 4°C. Mature zygotic embryos were excised from the seeds and placed on ½LM medium (with ½ of full concentrations of media recommended by Litvay et al. 1985) to initiate ET. We tested the influence on ET initiation frequency of nine combinations of growth regulators added to the medium:

A) 9 μM 2.4-D + 2.2 μM BA, abbreviated as 2.4-D/BA2.2
B) 9 μM 2.4-D + 4.5 μM BA, abbreviated as 2.4-D/BA4.5
C) 9 μM 2.4-D + 8.8 μM BA, abbreviated as 2.4-D/BA8.8
D) 9 μM NAA + 2.2 μM BA, abbreviated as NAA/BA2.2
E) 9 μM NAA + 4.5 μM BA, abbreviated as NAA/BA4.5
F) 9 μM NAA + 8.8 μM BA, abbreviated as NAA/BA8.8
G) 9 μM picloram + 2.2 μM BA, abbreviated as picloram/BA2.2
H) 9 μM picloram + 4.5 μM BA, abbreviated as picloram/BA4.5
I) 9 μM picloram + 8.8 μM BA, abbreviated as picloram/BA8.8

Combinations B, E and H (9 μM auxin and 4.5 μM BA) can be considered as control treatments because in previous experiments, we obtained the best results for ET initiation and proliferation when using these combinations (data not shown). All of the media were also supplemented with sucrose (10 g · dm^{-3}) and solidified with Phytagel (5 g · dm^{-3}; Sigma-Aldrich, Poznań, Poland). The pH of the media was 5.8. The explants were incubated at 22±1°C in darkness.

The following samples of plant material (fresh weight) were collected for guaiacol peroxidase activity analysis: mature zygotic embryos excised from seeds (which were the source explants), 8-week-old non-embryogenic calluses (NC), embryogenic calluses (EC), in which originated from explants, and embryogenic tissue (ET) from EC (Fig. 1). The samples consisted of 2–5 explants in three repeats for each growth regulator combination.

ET proliferation and peroxidase activity

Following ET initiation, we collected several lines of tissues to be proliferated in ½LM medium supplemented with 9 μM picloram and 4.5 μM BA, which is routinely used for this purpose in our laboratory. ET was incubated at 22±1°C in darkness. After two months of proliferation, we selected the two best-growing lines (one for *Picea abies* and one for *P. omorika*), and we tested the influence of the same nine growth regulator combinations as described previously, added into ½LM medium, on ET weight on days 7, 14 and 21 of culture. Finally, on day 21 of culture, we measured peroxidase activity in ET treated with these growth regulator combinations. Clumps of ET from each growth regulator combination treatment were collected for these analyses; three repeats were performed.

Results

ET initiation and peroxidase activity

Initiation of ET from mature zygotic embryos of both spruce species was achieved, irrespective of the growth regulator combinations added to the ½LM medium (Table 1). The highest ET initiation frequency in *Picea abies* was 13.00% for picloram/BA4.5, whereas in *P. omorika*, it was 12.22% for 2.4-D/BA8.8. The lowest ET initiation was obtained for the combination NAA/BA8.8 in both spruce species. There were no statistically significant differences in the ET initiation frequency for *Picea abies* and *P. omorika* explants cultured in the presence of various BA concentrations with various auxin types.

In the mature zygotic embryos of both spruce species used to initiate the embryogenic cultures, very low peroxidase activity was detected (0.0005 ± 0.0 units/FW; data not shown). The level of peroxidase activity in 8-week-old explants of both spruce species increased and varied depending on the growth regulator combinations added to the initiation medium (Fig. 2). In *Picea abies*, the activity was the highest for explants treated with 2.4-D/BA2.2 (Fig. 2a). The lowest activity of peroxidase was noted for picloram/BA8.8 (Fig. 2c). In 8-week-old *P. omorika* calluses, high peroxidase activity was observed under the 2.4-D/BA8.8 treatment (Fig. 2a). A low activity of the enzyme was noted for NAA/BA4.5 and NAA/BA8.8 (Fig. 2b) as well as picloram/BA2.2 and picloram/BA4.5 (Fig. 2c).

The differences in peroxidase activity between embryogenic and non-embryogenic calluses (EC and NC) after 8 weeks of culture were usually low, regardless of the growth regulator combinations (Fig. 2). However, in some cases, significant differences in peroxidase activity were observed between EC and NC of both spruce species treated with the same growth regulator combinations (Fig. 2).

In general, peroxidase activity in *Picea abies* calluses (NC and EC) ranged from approximately 50 to 280 units (Fig. 2), while for *P. omorika* explants, it was lower and ranged from 15 to 150 units (Fig. 2), regardless of the ability of the calluses to form ET. The greatest difference in peroxidase activity was detected between *P. abies* calluses (EC and NC) for NAA/BA8.8. The difference reached the level of 175.7 units (Fig. 2b). Significant differences in peroxidase activity was also observed in case of calluses (NC and EC) growing in the presence of 2.4-D/BA2.2 (Fig. 2a) and picloram/BA4.5 (Fig. 2c). These differences were 135.9 and 152.2 units, respectively. Peroxidase activity was higher for NC than for EC in these cases (Fig. 2a,c). Additionally, for these growth regulator combinations, the highest frequencies were obtained for the initiation of ET from ma-

Table 1. Embryogenic tissue (ET) initiation from mature zygotic embryos of *Picea abies* and *P. omorika* in ½LM medium supplemented with various growth regulator combinations

Species	Auxin (9 μM)	BA (μM)	Final number of explants	Zygotic embryos with ET (%)	χ^2 *P*
Picea abies	2.4-D	2.2	*n*=50	10.00±0.30	0.17
		4.5	*n*=50	8.00±0.27	0.9208
		8.8	*n*=50	8.00±0.27	
	NAA	2.2	*n*=50	4.00±0.20	4.79
		4.5	*n*=50	12.00±0.33	0.0908
		8.8	*n*=50	2.00±0.14	
	picloram	2.2	*n*=50	6.00±0.24	1.43
		4.5	*n*=46	13.00±0.34	0.4882
		8.8	*n*=46	8.70±0.28	
P. omorika	2.4-D	2.2	*n*=41	2.44±0.16	3.36
		4.5	*n*=50	8.00±0.27	0.1863
		8.8	*n*=49	12.22±0.33	
	NAA	2.2	*n*=50	8.00±0.27	3.36
		4.5	*n*=49	10.20±0.31	0.1860
		8.8	*n*=50	2.00±0.14	
	picloram	2.2	*n*=50	4.00±0.20	0.29
		4.5	*n*=50	4.00±0.20	0.8656
		8.8	*n*=50	6.00±0.24	

Data are means ± SD of two experiments consisting of five replicates each, n=50. Final number of explants = explants not contaminated. 2.4-D – 2.4-dichlorophenoxyacetic acid; NAA – 1-naphtaleneacetic acid; picloram – 4-amino-3.6.6-trichloropicolinic acid; BA – 6-benzyladenine; ET – embryogenic tissue

ture zygotic embryos, which were 10.00% and 13.00%, respectively (Table 1).

The greatest differences in peroxidase activity between *Picea omorika* calluses (NC and EC) were observed for NAA/BA2.2 or NAA/BA8.8 (Fig. 2b) and 2.4-D/BA2.2 (Fig. 2a). The differences were 81.9, 45.3 and 40.4 units, respectively (Fig. 2). In all of these cases, the peroxidase activity associated with NC was higher than for EC (Fig. 2a, b). However, the ET initiation frequencies for these growth regulator combinations were 8.00%, 2.00% and 2.44%, respectively, and did not reach the highest values, in contrast to what was observed for the Norway spruce (Table 1). The highest ET initiation frequency from mature zygotic embryos of *P. omorika* was noted for 2.4-D/BA8.8 (12.22%) and NAA/BA4.5 (10.20%). However, the differences in peroxidase activity between *P. omorika* calluses (NC and EC) were very low under these growth regulator combinations, at 7.4 (Fig. 2a) and 7.3 units (Fig. 2b), respectively. It must be emphasized that the highest ET initiation frequencies for this spruce species were associated with both

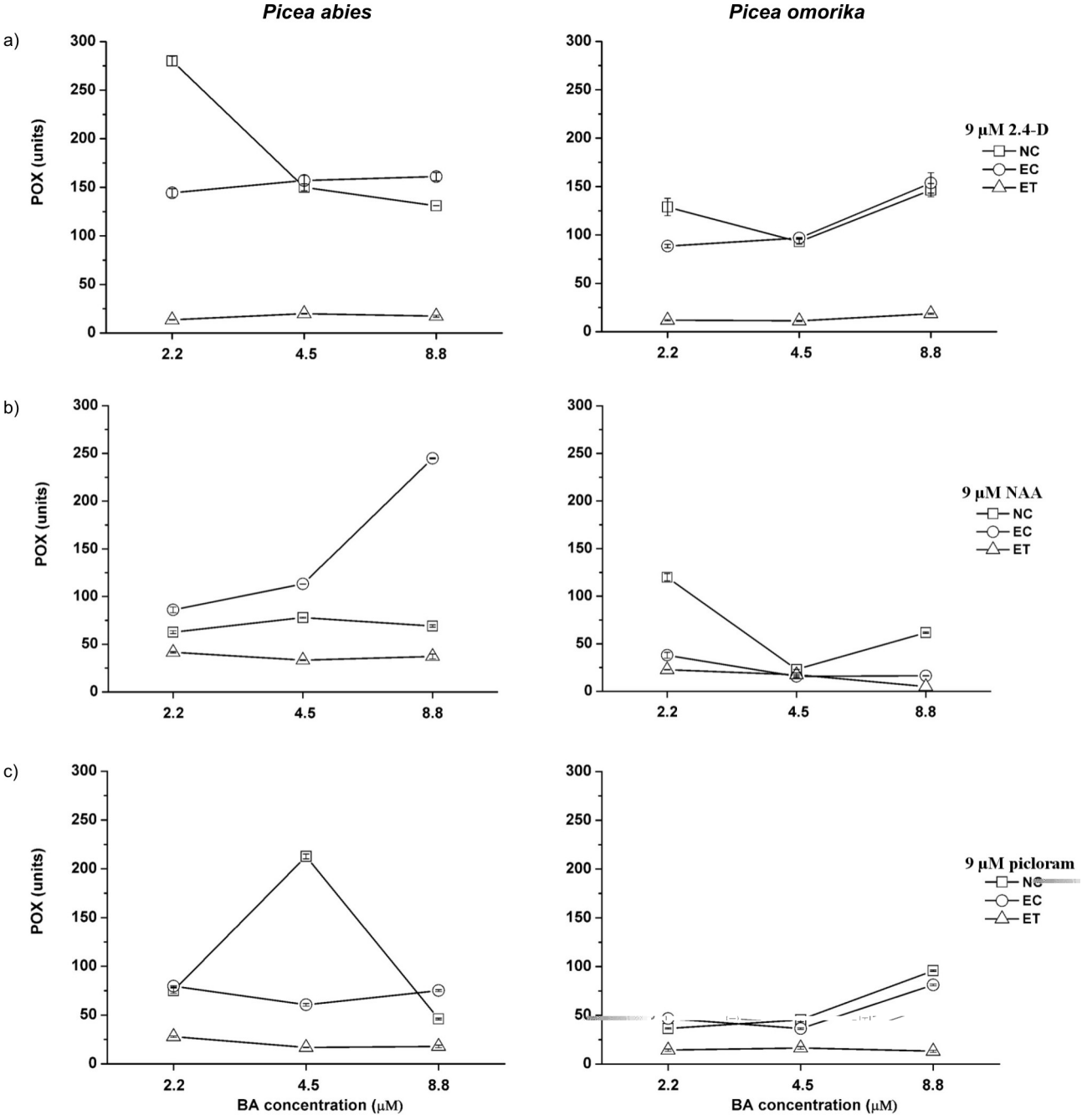

Fig. 2. Peroxidase (POX) activity in *Picea abies* and *P. omorika* 8-week-old callus (NC), embryogenic callus (EC) and embryogenic tissue (ET) cultured in ½LM medium supplemented with various BA concentration and a) 9 μM 2.4-D, b) 9 μM NAA, c) 9 μM picloram. 1 unit=1nkatmin^{-1}mgprot^{-1}. Means ± SD, n=3

high and low peroxidase activities in NC and EC (Table 1, Fig. 2a, b).

The activity of peroxidase in the newly initiated embryogenic tissue (ET) from 8-week-old calluses of *Picea abies* and *P. omorika* was generally lower than in the callus itself (EC), regardless of the auxin type and BA concentrations added to the initiation medium (Fig. 2). The activity level of this enzyme was the same between ET and the callus from which it was generated only in *P. omorika* when the initiation medium was supplemented with NAA/BA4.5 (Fig. 2b).

ET proliferation and peroxidase activity

ET proliferation was achieved for both Norway and Serbian spruce for all growth regulator combinations. However, the ET weight was generally lower for *Picea abies* compared to *P. omorika* ET on days 14–21 of culture (Fig. 3). The highest *P. abies* ET weight of 2.23 g, observed on day 21 of culture, was achieved in the media supplemented with picloram and BA at concentration 2.2 μM (Fig. 3c), whereas the lowest weight of 1.48 g was observed under the 2.4-D/BA8.8 treat-

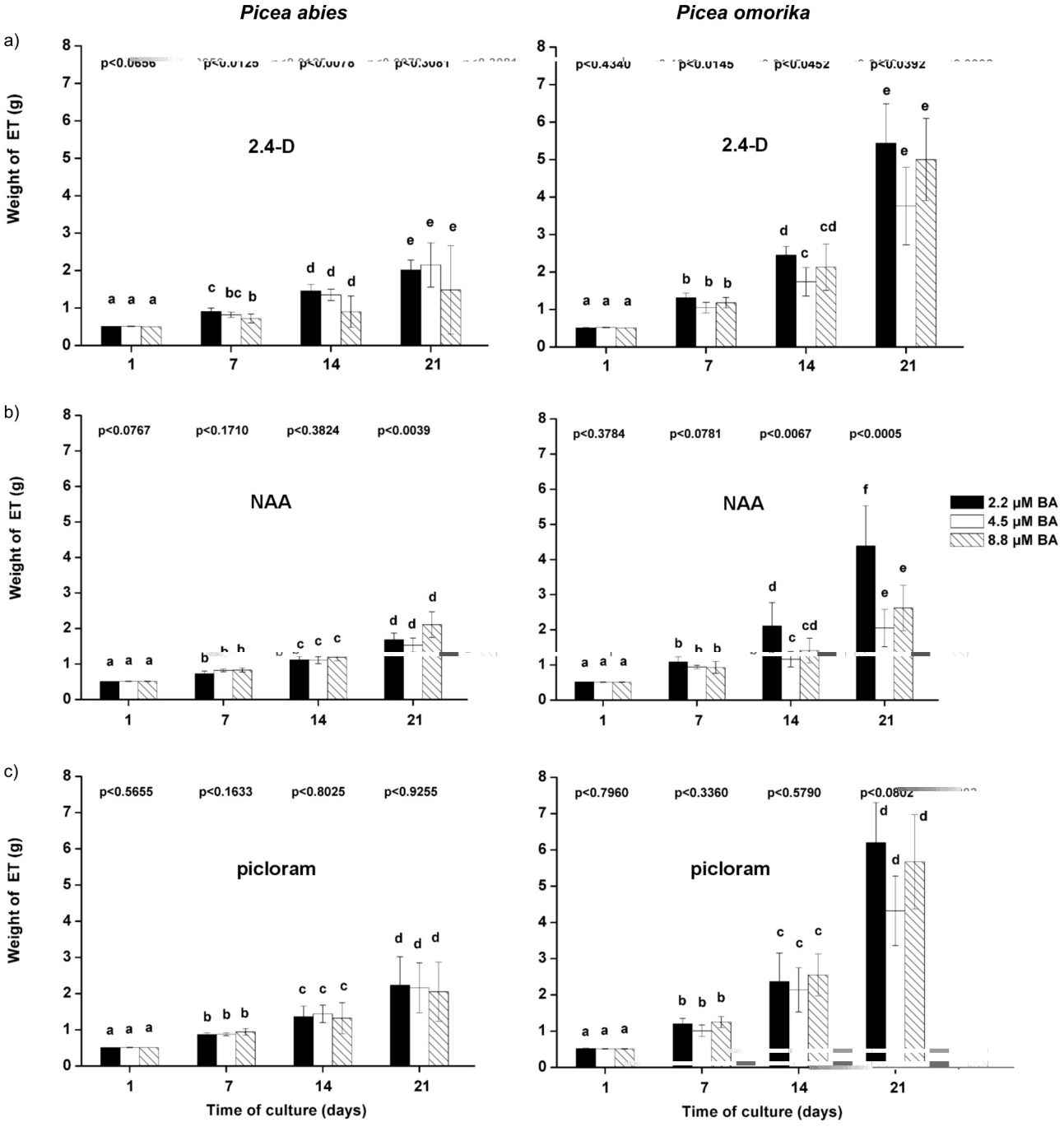

Fig. 3. *Picea abies* and *P. omorika* embryogenic tissue (ET) weight during 21 days of culture in ½LM medium supplemented with a) 9 μM 2.4-D, b) 9 μM NAA, c) 9 μM picloram and following concentrations 2.2 μM, 4.5 μM, 8.8 μM of BA in each variant. Means ± SD, n=6

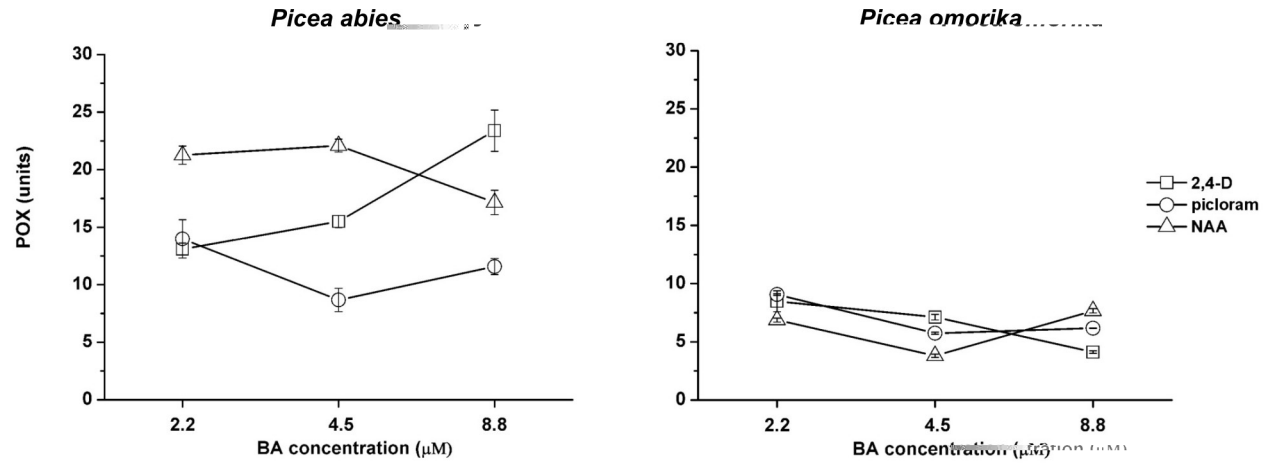

Fig. 4. Effect of hormone combinations on peroxidase (POX) activity in *Picea abies* and *P. omorika* embryogenic tissue (ET) proliferated for 1–21 days in ½LM medium with 9 μM 2.4-D, NAA, picloram and 2.2 μM – 8.8 μM BA. Means ± SD, n=3

ment (Fig. 3a). On day 21 of culture, no statistically significant differences in *P. abies* ET proliferation were detected.

The highest *P. omorika* ET weight, on day 21 of culture, was usually obtained in the medium supplemented with 2.2 μM BA, irrespective of the type of auxins added to the proliferation medium (Fig. 3). It ranged from 4.39 g for NAA (Fig. 3b) to 6.20 g for picloram (Fig. 3c). The lowest ET weight was observed in the medium supplemented with NAA/BA4.5 (Fig. 3b). Statistically significant differences in *P. omorika* ET proliferation on day 21 of culture were detected under combinations of NAA with various BA concentrations (Fig. 3b).

On day 21 of culture, varied peroxidase activity levels were found in ET for both of the tested spruce species exposed to the specific growth regulator combinations. The peroxidase activity in *P. abies* ET was usually higher (more than 10 units) than in *P. omorika* ET (Fig. 4). The highest peroxidase activity in *P. abies* ET was found in the medium supplemented with 2.4-D/BA8.8 (Fig. 4). The lowest activity was observed in ET cultured with picloram/BA4.5 (Fig. 4). When the proliferation rates and peroxidase activities were compared for *P. abies* ET, on day 21 of culture, we noticed a trend of more rapid growth of tissue at a lower activity level of this enzyme (Fig. 3, Fig. 4). This was particularly evident for growth regulator combinations including 2.4-D (Fig. 3a, Fig. 4) or NAA (Fig. 3b, Fig. 4). The highest peroxidase activity in *P. omorika* ET was detected under the picloram/BA2.2 treatment (Fig. 4), while the lowest was associated with NAA/BA4.5 (Fig. 4). When the proliferation rates and peroxidase activities in *P. omorika* ET were compared on day 21 of culture, we observed a tendency to a better growth of the tissue when peroxidase activity was higher in most cases, in contrast to what was found for *P. abies* (Fig. 3, 4). An exception to this trend was observed for the culture of ET in the presence of 8.8 μM BA and 2.4-D or picloram, where we observed a similar trend as in *P. abies* (Fig. 3a, c).

Embryogenic potential of the tested lines

Both the *Picea abies* and *P. omorika* ET lines presented the ability to produce somatic embryos. The *P. abies* lines regenerated 754 embryos/g fresh weight of embryogenic tissue on average, of which 92 were cotyledonary embryos (Table 2). *P. omorika* ET showed a lower embryogenic potential, regenerating 156 embryos/g fresh weight of embryogenic tissue on average, including 3 cotyledonary embryos. The statistical analysis (the Tukey test) showed significant differences in the embryogenic potential between the tested lines of the two spruce species.

Table 2. Number of somatic embryos (per 1 g fresh weight of embryogenic tissue) after 5 weeks of incubation in maturation medium

Species	Total embryos	Cotyledonary embryos
Picea abies	754a±181.97	92a±30.51
P. omorika	156b±47.52	3b±1.53
P	0.0053	0.0072

Data are means ± SD of three experiments consisting of three replicates each. Means followed by the various letter within column are significantly different (Tukey's test, P=0.05)

higher for *Picea omorika* than for *P. abies* (Fig. 3). However, the best ET growth was generally obtained using a lower BA concentration (2.2 μM), regardless of the type of auxin used. This situation was more evident for *P. omorika* (Fig. 3).

This research showed higher peroxidase activity in *P. abies* ET than in *P. omorika* ET (Fig. 4). Significant differences in the activity of this enzyme may be due to the different spruce species tested or different sensitivities of the tested embryogenic lines to the growth regulators used in the proliferation medium. In most cases, opposite trends were observed for the ET proliferation rate and peroxidase activity in the two spruce species, depending on the auxin type and BA concentration added to the medium. *Picea abies* ET presented more intensive growth at a lower peroxidase activity level, whereas *P. omorika* exhibited increased growth at a higher activity level of this enzyme. The exception to this trend was ET treated with 8.8 μM BA, in which the trend was similar to that of *P. abies*. Thus, the combination of growth regulators affects the level of peroxidase activity in proliferating embryogenic tissues and, consequently, the intensity of tissue growth and the process of somatic embryogenesis. The different reactions of ET of the two spruce species to changes in the peroxidase activity level might result from the different abilities of the tested ET lines to form somatic embryos, with *Picea abies* tissue being found to be more efficient than that of *P. omorika* with respect to this process (Table 2). The embryogenic tissue of coniferous species do not present a homogenous structure but consists of so-called proembryos at several stages of development (Filonova et al. 2000). Only some of the most developed proembryos transform into somatic embryos. Hence, the peroxidase activity level in ET at the proliferation stage may be related to the number of the most developed proembryos present that are ready to initiate a new embryo development phase after the removal of auxin and cytokinin from the medium and treatment with abscisic acid (Stasolla et al. 2002).

The pattern of changes in guaiacol peroxidase activity observed in the present study in the induction phase of somatic embryogenesis in *Picea abies* and *P. omorika* indicate that this enzyme is related to this process.

The relationship between the growth regulator type added to the medium and the activity of peroxidase linked to somatic embryogenesis was more pronounced at the stage of proliferation than at the initiation stage of ET in both spruce species.

Conclusions

These studies presented the specificity effect of guaiacol peroxidase on somatic embryogenesis in the tested spruce species. We found that this peroxidase was active both in explants exposed to the chosen growth regulator systems and in embryogenic tissues. Guaiacol peroxidase activity was also detected in mature zygotic embryos used to establish the embryogenic cultures. In the two tested spruce species, the same pattern of guaiacol peroxidase activity was found, from the lowest level in explants to the highest in 8-week-old calluses. A moderate level was observed in the induced and proliferated embryogenic tissues, regardless of the composition of the growth regulators added to the culture media. The type and concentration of growth regulators used in the initiation and proliferation of embryogenic tissues had no statistically significant effect on peroxidase activity, although during the initiation often its increased level was observed in calluses treated with 2.4-D.

Detection of guaiacol peroxidase activity in the induction phase of somatic embryogenesis proves its participation in this process. The subsequent change in its activity indicate that this peroxidase can be a biochemical marker of somatic embryogenesis of the tested spruce species. But to confirm finally its role in this process, it is necessary to determine the pattern of guaiacol peroxidase in further studies.

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