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ORIGINAL PAPER

Adventitious rooting adjuvant activity of 1,3-di(benzo[*d*]oxazol-5-yl) urea and 1,3-di(benzo[*d*]oxazol-6-yl)urea: new insights and perspectives

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Abstract Here we report new insights on the adventitious rooting adjuvant activity of 1,3-di(benzo[*d*]oxazol-5-yl)urea (5-BDPU) and 1,3-di(benzo[*d*]oxazol-6-yl)urea (6-BDPU), both symmetrically substituted urea derivatives that do not show either auxin- or cytokinin-like activity per se. Our data demonstrate that these synthetic molecules enhance adventitious rooting in distantly-related herbaceous and woody species, in the presence of endogenous or exogenous auxin. For the first time, we report that BDPUs enhance adventitious rooting in the presence of either indole-3-butyric acid (IBA) or 1-naphtalene acetic acid and that their optimal concentration depends on the strength of the exogenous auxin. Trying to understand the mode of action of BDPUs, we also show that their adventitious rooting adjuvant activity correlates with high mRNA levels of auxin-responsive genes related to the adventitious rooting process at the very early stages of adventitious rooting, before the activation of cell divisions in pine hypocotyls cuttings. The high mRNA levels are measured in the presence of low auxin concentrations and BDPUs. The mRNA levels quantified in these conditions are similar to those measured in the presence of high auxin concentrations but in the absence of BDPUs. In addition, the spatial distribution of endogenous auxin is localized in globular-shaped structures of cell divisions located centrifugal to the resin canals, at the positions of adventitious root formation, in the presence of urea derivatives and IBA after 6 days of the root induction process.

Keywords Adventitious rooting · Auxin-signalling pathway · Auxin-spatial distribution · Distantly-related species · Urea derivatives

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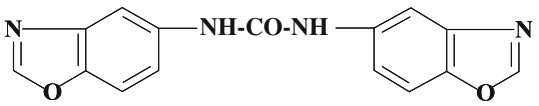
Introduction

Adventitious rooting is a complex physiological process which requires that groups of cells undergo a new developmental programme starting from the reactivation of cell division and further differentiation of dividing cells into roots, as adventitious roots arise from tissues in which they are normally absent. The rooting process depends on endogenous and exogenous factors, such as hormonal balance, tissue maturity or injuries, but different species respond in a different way to the same inductive stimulus and this correlates with different rooting capacity. In fact, based on their rooting abilities, plants can be roughly described as easy-to-root or difficult-to-root species, the woody plants being more recalcitrant than the herbaceous ones (Altamura 1996). An efficient rooting treatment, yielding a high quality root system, plays a key role in either in vivo or in vitro vegetative propagation programmes. The percentage of rooted shoots, the number of roots per rooted shoot and the absence of callus formation are the most important features that should be considered,

as they enhance the survival rate of vegetatively propagated plants (George 1993). It is well known that auxin plays a central role in adventitious root formation (Blakesley 1994), but sometimes, depending on concentration, exposure time and type of auxin, its effectiveness may vary or inhibitory side-effects may arise, causing poor rooting (De Klerk 1995; De Klerk et al. 1997, 1999; Fogaca and Fett-Neto 2005). Indole-3-acetic acid (IAA) was the first auxin to be used in stimulating adventitious roots (Cooper 1935) and soon after the root inducing capacity of newly synthesized compounds, indole-3-butyric acid (IBA), with an indolic ring as IAA, and 1-naphtalene acetic acid (NAA), with a naphthalenic ring replacing the indolic one, was discovered (Zimmerman and Wilcoxon 1935). Depending on the species, NAA shows a relatively high root inducing capacity, that, however, is often counterbalanced by the inhibition of the outgrowth of the previously induced root primordia (De Klerk 1995; Diaz-Sala et al. 1996; De Klerk et al. 1997). It has been demonstrated that NAA predominantly enters cells by diffusion, being rather lipophilic, and once inside the cells it is not destroyed by auxin-oxidase (Smulders et al. 1990; Delbarre et al. 1996). Recently, it has been reported that in tobacco BY-2 cells NAA is almost immediately converted into metabolites, naphtalene-1-acetic acid-glucosyl-ester (NAA-Glc) being the major one, that remain trapped inside the cells, as they are unable to translocate across the plasma membrane via auxin efflux carriers (Hošek et al. 2012). IBA, originally classified as a synthetic auxin, has been definitively recognized as an endogenous plant compound (Epstein and Ludwig-Müller 1993; Ludwig-Müller 2000; Bartel et al. 2001), now worldwide used for commercial rooting of many species (Hartmann et al. 1997).

Despite the endogenous auxin accumulation at the wounded site of cuttings, due to its basipetal polar transport, and the exogenous application to in vivo or in vitro propagated cuttings, rooting recalcitrance is still a limiting factor for the propagation programs of many species (Diaz-Sala et al. 1996). Many efforts have been made to enhance rooting, investigating the effect of other plant growth regulators (Haissig and Davis 1994), the effect of different in vitro culture conditions (etiolation, culture medium pH, etc.; George 1993), or the effect of *Agrobacterium*-mediated gene transfer (Falasca et al. 2000; Welander et al. 2009 and references therein). Moreover, a wide range of chemicals have been combined with auxin to avoid the abnormal growth or the deleterious effects of the auxin treatment, that sometimes occur, and to achieve a high quality root system (Welander and Huntrieser 1981; James 1983; Cheng et al. 1992; Orlikowska 1992; Rugini et al. 1993; Pawlicki and Welander 1995; Auderset et al. 1996, 1997; Kevers et al. 1997; Diaz-Sala et al. 2002; Tamimi 2003). On this point, we have reported that two urea derivatives, namely the *N*,*N*′-bis-(2,3-methylenedioxyphenyl)urea (2,3-MDPU) and the *N*,*N*′-bis-(3,4-methylenedioxyphenyl)urea (3,4-MDPU), showing neither auxin- nor cytokinin-like activity per se, may be used as adventitious rooting adjuvants in the presence of endogenous or exogenous auxin (Ricci et al. 2001, 2003), and an interaction with the auxin-signalling pathways in pine rooting competent cuttings has been described (Ricci et al. 2008). The enhancement of adventitious root formation was strictly related to the symmetrical presence of two methylenedioxyphenyl groups on the urea matrix of MDPUs. In the attempt to better understand the structure–activity relationship of these urea derivatives, a chemical modification of the aromatic moiety of MDPUs consisting in a replacement of the methylenedioxyphenyl groups by the isostere benzoxazole ones was performed (Ricci et al. 2006). The two newly synthesized compounds, namely the 1,3-di(benzo[*d*]oxazol-5-yl)urea (5-BDPU) and the 1,3-di(benzo[*d*]oxazol-6-yl)urea (6-BDPU), showed a conserved adventitious rooting enhancement in apple and mung bean systems, without either auxin- or cytokinin-like activity per se (Ricci et al. 2006).



1,3-di(benzo[*d*]oxazol-5-yl)urea
5-BDPU

1,3-di(benzo[*d*]oxazol-6-yl)urea
6-BDPU

Fig. 1 Molecular structure of the diphenylurea derivatives of which the biological activity has been investigated

These data led us to hypothesize the existence of a new category of adventitious rooting adjuvants, i.e. symmetrical urea derivatives showing similar chemical structure, interacting with endogenous or exogenous auxin, without being auxin synergists. Here we analyze the effect of both 5-BDPU and 6-BDPU (Fig. 1) on the adventitious rooting capacity of distantly-related herbaceous and woody species, their cooperation with endogenous auxin and with different exogenous auxins as well as their interaction with auxin-signalling pathways. Moreover, with the aim of understanding if and how these urea derivatives affect the auxin local pools at the wounded site of cuttings, the spatial distribution of endogenous IAA in the presence of 5-BDPU or 2,3-MDPU, as representative samples of this new category of adventitious rooting adjuvants, has been investigated.

Materials and methods

Preparation of chemical solutions

The 1,3-di(benzo[*d*]oxazol-5-yl)urea (5-BDPU), the 1,3-di(benzo[*d*]oxazol-6-yl)urea (6-BDPU) and the *N*,*N*′-bis-(2,3-methylenedioxyphenyl)urea (2,3-MDPU) were dissolved in dimethylsulfoxide (DMSO) as well as the NAA. The final concentration of DMSO in the in vitro culture medium or in the aqueous solutions did not exceed the one considered toxic (0.2 %) (Schmitz and Skoog 1970). The 5-BDPU, the 6-BDPU and the 2,3-MDPU, synthesized as previously reported (Ricci et al. 2001, 2006), were of analytical grade. The aqueous solutions of indole-3-butyric acid (IBA) and benzylaminopurine (BAP) were sterilized by filtration.

Adventitious rooting of Arabidopsis seedlings

Plant material

Arabidopsis thaliana ecotype Columbia (Col-0) seeds were surface sterilized in 70 % ethanol for 1 min, followed by 10 min in 50 % commercial bleach (equivalent to 2.5 % NaOCl), washed five times in sterile distilled water, sown on ¼ strength Murashige and Skoog (MS) medium (Murashige and Skoog 1962) supplemented with 0.8 % (w/v) agar, pH 5.8 (germination medium) and kept in the dark at 26 °C.

Adventitious rooting induction

Groups of 10 three-day-old etiolated seedlings were transferred to Petri dishes containing full strength MS medium, supplemented with MS vitamins, 3 % (w/v) sucrose, 0.8 % (w/v) agar, pH 5.8, under different rooting conditions. The medium was supplemented with 1, 2, 4 or 8 μM 5-BDPU or 6-BDPU alone and in the simultaneous presence of 0.1 μM IBA or 0.1 μM NAA, as auxins. Medium containing DMSO or 0.1 μM IBA or 0.1 μM NAA were used as controls. Petri dishes were placed vertically in a growth chamber at light intensity of 27 μmol/m^2/s at 26 °C under 16 h photoperiod (standard conditions from now on). Emergent adventitious roots on the hypocotyls were scored at 7 days after transfer to the light using a stereomicroscope. The experiments were done in triplicate and repeated twice.

Histochemical localization of GUS activity

Arabidopsis thaliana DR5::GUS (Ulmasov et al. 1997) transgenic plants in Col-0 background, homozygous for the reporter gene, were used for this study. Seeds were surface sterilized as reported above, sown on the germination medium supplemented with 1 % (w/v) sucrose and 50 μg/mL kanamycin, then cultured in a growth chamber at standard conditions. Four-day-old seedlings were transferred to ½ strength MS liquid medium plus 1.5 % (w/v) sucrose, alone (hormone free, HF) or supplemented with : 10 or 100 nM IBA or NAA alone as exogenous auxins; 10, 100 nM, 1, 10 or 100 μM 5-BDPU alone; IBA (10 or 100 nM) in combination with 5-BDPU (10 nM or 100 μM); NAA (10 or 100 nM) in combination with 5-BDPU (10 nM or 100 μM). Ten seedlings were used in each treatment. The HF condition was assumed as internal control to validate the experimental procedure, while the supplementation of both IBA and NAA was assumed as control of 5-BDPU and each combination treatment, respectively.

After 5 h incubation in multiwell dishes kept in a growth chamber at standard conditions, the seedlings were vacuum infiltrated for 10 min and then submerged in X-Gluc solution containing 100 mM sodium phosphate buffer (pH 7.0), 1 mM 5-bromo-4-chloro-3-indolyl-β-D-glucuronide, 10 mM EDTA, 1 mM K3/K4(FeCN)$_6$, 0.1 % (v/v) Triton X-100, 1 % (v/v) *N*,*N*-dimethylformamide (Bai and De-Mason 2008, with minor modifications). Incubation was carried out for 5 h at 37 °C. The seedlings were then washed three times in distilled water to remove excess substrate, cleared in freshly made ethanol:acetic acid (6:1, v/v) solution overnight at 4 °C, washed twice in 96 % (v/v) ethanol and stored in 70 % (v/v) ethanol (Mattsson et al. 2003, with minor modifications). Seedlings were observed for each treatment with a stereomicroscope with Nikon DS-Fil digital camera (whole seedling) and with a conventional light microscope with a Leica DC 100 digital camera (close-up views). Experiments were repeated three times.

Adventitious rooting of apple stem slices

Plant material

In vitro shoot cultures of *Malus pumila* Mill. rootstock M26 were maintained as previously described (Ricci et al. 2006) with minor modifications. The cuttings deprived of apices were propagated on a micropropagation medium (MS salts, plus 0.4 mg/L thiamine HCl, 0.5 mg/L nicotinic acid, 0.5 mg/L pyridoxine HCl, 100 mg/L myo-inositol, 10 g/L sucrose, 20 g/L sorbitol, 0.65 % (w/v) agar, 5.8 pH). The medium was supplemented with 1.3 μM BAP and 0.25 μM IBA. After a 5-week incubation stage at standard conditions, clusters consisting of 4–6 shoots were formed by axillary branching. Individual shoots, 2–2.5 cm in length, were used either for further propagation or for preparing 1-mm thick stem slices.

Adventitious rooting induction

Stem slices were prepared and distributed over different rooting treatments as previously reported (Ricci et al. 2006). Groups of 16 slices were cultured in Petri dishes with the apical side on a nylon mesh put on the above described culture medium supplemented with 1, 2, 4 or 8 μM 5-BDPU in the simultaneous presence of 1 μM NAA, as auxin. The dishes were incubated upside down in the darkness for 6 days. Simply removing the nylon mesh, all the slices were then transferred to fresh culture medium lacking of 5-BDPU and NAA; the dishes were incubated at standard conditions. Control was performed with stem slices cultured in the presence of 1 μM NAA alone and slices were subjected to the same manipulations described above. The number of rooted slices in relation to total slices in specific treatments and the induced number of roots were counted after 14 days. The percentage of rooting was expressed as a percentage of the control (1 μM NAA) by the formula (Tested-Control/Control × 100) [(T-C/C) × 100]. All the experiments were carried out in triplicate and repeated twice.

Adventitious rooting of Pinus cuttings

Plant material

Pinus radiata D. Don seeds were provided by local growers (Vitoria, Spain) and kept at 4 °C until used. The seeds were soaked in running tap water for 12 h and then sown in fine wet vermiculite. Germination and seedling growth occurred in a growth chamber at light intensity of 27 μmol/m^2/s under a 16 h photoperiod. Light/dark temperatures were 25 and 21 °C, respectively. The seedlings were watered daily with distilled water.

Adventitious rooting induction

Pinus radiata hypocotyl cuttings from 21-day-old seedlings were prepared as previously described by severing the hypocotyl at its base to a length of 2.5 cm (Ricci et al. 2008). Groups of five hypocotyl cuttings were cultured in vials containing 20 mL of aqueous solutions of 5-BDPU or 6-BDPU alone at 0.1, 1 or 10 μM, or in combination with 0.1, 1 or 10 μM IBA or NAA, as exogenous auxins. Distilled water, solutions of IBA alone or NAA alone at the same different concentrations (0.1, 1 or 10 μM) and a solution containing DMSO alone were used as controls. The vials were incubated at light intensity of 27 μmol/m^2/s at 24 °C under a 16 h photoperiod and they were refilled daily with distilled water to replace water lost to evapotranspiration. All the experiments were carried out in triplicate and repeated twice. Rooting was analyzed as percentage of rooted cuttings and as the number of roots per rooted cutting. Results were taken after 28 days of culture.

Histological analysis of rooting

For histological analysis of adventitious root formation in the presence of 5-BDPU and IBA, basal 5 mm segments of hypocotyl cuttings from 21-day-old seedlings treated with 10 μM 5-BDPU, 1 μM IBA, 10 μM 5-BDPU plus 1 μM IBA, or with distilled water, as control, were fixed in formalin-acetic acid-alcohol (FAA), dehydrated in a tertiary-butyl-alcohol series, gradually embedded in paraffin, transversely sectioned at 10 μm thickness with a rotary microtome (Reichert-Jung 2040) and stained with safranin-fast green (Berlyn and Miksche 1976). Hypocotyl cuttings were sampled at the beginning of the rooting experiment (time 0), after 1, 2, 4, 6, 10 and 13 days, for each treatment.

RNA extraction, cDNA synthesis and quantitative RT-PCR (qRT-PCR)

Analyses were carried out as previously described (Ricci et al. 2008). For qRT-PCR, a 18S rRNA gene (Ri18S) was used as internal control. Primers were designed based on the sequence of *Pinus wallichiana* 18S rRNA (gi: 403026), and confirmed in *P. radiata*. *PrSCL1* (accession number DQ683567) and *PrSHR* (accession number EU044786) specific primers were designed based on the *P. radiata* full-length cDNA sequences previously obtained (Sanchez et al. 2007; Solé et al. 2008). Expression ratios to time 0 were obtained from the equation $2^{-\Delta\Delta CT}$ (Applied Biosystems, Technical Bulletin #2 P/N4303859B). Results are expressed as mean values ± standard error from three biological replicates. For statistical analysis comparison between groups was done by Students' *t* test and comparison among multiple groups was done by ANOVA. Results were considered to be statistically significant at $p < 0.01$.

Primers:

18S rRNA

Ri18StrFor 5'-GCGAAAGCATTTGCCAAGG-*3*'
Ri18StrRev 5'-ATTCCTGGTCGGCATCGT TTA-3'

SCARECROW-LIKE gene

PrSCL1trFor 5'-TCAATGTCTGGCAAATCGTCC-*3*'
PrSCL1trRev 5'-CGCCCAGTCTCTTCAATTCT-*3*'

SHORT-ROOT gene

PrSHRtrFor1 5'-GAACCAGTGCAAGGAGCATTG-*3*'
PrSHRtrRev1 5'-AAATCCTGCCTCCTTGAGCCT-*3*'

Auxin immunolocalization

Auxin immunolocalization was performed as described by Prem et al. (2012). One-cm basal segments of the hypocotyls treated with auxin combined with 5-BDPU or 2,3-MDPU, and the corresponding controls, were excised and fixed in 4 %

paraformaldehyde in phosphate-buffered saline (PBS) at 4 °C overnight. The segments were then washed three times in PBS, 10 min each, and post-fixed in 0.1 % paraformaldehyde in PBS at 4 °C until use. Cryosections were incubated with 5 % bovine serum albumin (BSA) in PBS for 5 min, and then incubated with anti-indole-3-acetic acid (IAA) mouse monoclonal antibodies (Sigma-Aldrich, St. Louis, MO, USA), 1:100 dilution in 1 % BSA, overnight at 4 °C in wet chamber. After washing in 1 % BSA five times, 5 min each wash, the signal was revealed with ALEXA 568 conjugated anti-mouse antibodies (Molecular Probes, Eugene, Oregon, USA), 1:25 dilution in PBS, for 45 min in the dark. After washing in PBS, the sections were counterstained with DAPI, mounted in Mowiol and observed in a Leica SP5 confocal microscope. Confocal optical sections were collected using LAS AF confocal scanning. Controls were performed by replacing the first antibody with PBS.

Results

Effect of BDPUs on adventitious rooting of Arabidopsis seedlings

The effect of BDPUs on adventitious rooting in *Arabidopsis* was tested in the absence of the exogenous auxin, or supplementing the medium with either IBA or NAA. Rooting was achieved from hypocotyls of Col-0 seedlings cultured on medium containing increasing concentration of 5-BDPU or 6-BDPU alone. The mean root number obtained in the presence of 4 μM 5-BDPU or 6-BDPU (3.1 and 3.3 respectively) or in the presence of 8 μM 5-BDPU or 6-BDPU (4.2 and 4.3 respectively) was significantly higher (Student's *t* test) than that obtained in the presence of control solvent DMSO (2.5) (Table 1A). A similar result was obtained when the seedlings were cultured in the presence of 5-BDPU or 6-BDPU in combination with 0.1 μM IBA. The mean root number obtained in the presence of 4 μM 5-BDPU or 6-BDPU plus 0.1 μM IBA (3.9 and 4.4 respectively) or in the presence of 8 μM 5-BDPU or 6-BDPU plus 0.1 μM IBA (4.2 and 4.3 respectively) was significantly higher (Student's *t* test) than that obtained in the presence of 0.1 μM IBA alone (3.0) (Table 1B). The result was consistently different when the seedlings were cultured in the presence of 5-BDPU or 6-BDPU in combination with 0.1 μM NAA. In fact, only in the presence of 2 μM 5-BDPU or 6-BDPU plus 0.1 μM NAA the mean root number was significantly higher (Student's *t* test) than that obtained in the presence of 0.1 μM NAA alone (4.1 or 4.6 respectively vs. 3.3) (Table 1C).

Table 1 Adventitious rooting of *Arabidopsis* seedlings

(μM)	0	1	2	4	8
(A)					
5-BDPU	2.5 ± 0.15	2.9 ± 0.39	2.9 ± 0.27	3.1* ± 0.23	4.2* ± 0.31
6-BDPU	2.5 ± 0.15	2.4 ± 0.26	2.9 ± 0.22	3.3* ± 0.33	4.3* ± 0.33
(μM)	0.1 μM IBA plus				
	0	1	2	4	8
(B)					
5-BDPU	3.0 ± 0.13	3.4 ± 0.32	3.1 ± 0.30	3.9* ± 0.33	4.2* ± 0.38
6-BDPU	3.0 ± 0.13	3.5 ± 0.33	3.1 ± 0.31	4.4* ± 0.27	4.3* ± 0.38
(μM)	0.1 μM NAA plus				
	0	1	2	4	8
(C)					
5-BDPU	3.3 ± 0.17	3.3 ± 0.27	4.1* ± 0.27	3.4 ± 0.23	3.2 ± 0.32
6-BDPU	3.3 ± 0.17	3.3 ± 0.29	4.6* ± 0.41	3.7 ± 0.28	3.2 ± 0.31

The number of root was counted after 7 days

(A) Mean root number ± SE obtained in the presence of 1, 2, 4, 8 μM of 5-BDPU or 6-BDPU supplemented alone in the culture medium. Control was performed with DMSO alone (0 μM)

(B) Mean root number ± SE obtained in the presence of 1, 2, 4, 8 μM of 5-BDPU or 6-BDPU in the simultaneous presence of 0.1 μM IBA. Control was performed with 0.1 μM IBA alone

(C) Mean root number ± SE obtained in the presence of 1, 2, 4, 8 μM of 5-BDPU or 6-BDPU in the simultaneous presence of 0.1 μM NAA. Control was performed with 0.1 μM NAA alone

* Significantly different (Student's *t* test); n = 6

Effect of BDPUs on GUS activity in *DR5::GUS* transgenic plants

The synthetic auxin response reporter construct *DR5::GUS* has become a useful tool to localize auxin responsiveness regions *in planta*. GUS expression was detected at root tips in all plants tested and at cotyledon tips in specific plants, as already described (Ulmasov et al. 1997; Sabatini et al. 1999; Benková et al. 2003; Bai and DeMason 2008; Mugdil et al. 2009), at day four after sowing when seedlings were cultured in HF conditions (data not shown). No considerable changes in this spatial pattern were detected when seedlings were cultured in the presence of the different 5-BDPU concentrations (data not shown). Interestingly, GUS expression was strengthened when 5-BDPU was supplemented to seedlings simultaneously with exogenous auxins. In fact, GUS activity was clearly induced and restricted to root tips in the presence of exogenous 10 nM IBA, while a slight colouring randomly located occurred along the primary root when 10 nM 5-BDPU or 100 μM 5-BDPU were also present (Fig. 2A). When tenfold higher IBA concentration was supplemented to the seedlings, staining was detectable at the root tips; while, the DR5 signal was extended along the primary root and even in the central cylinder of roots in the presence of both, IBA and 5-BDPU, exogenously applied mixtures (Fig. 2B). NAA induced very high GUS expression when compared with the results obtained in the presence of exogenous IBA. GUS staining was darker and it was shown either at root tips or extended to the primary root when the seedlings were treated with different concentrations of 5-BDPU in combination with NAA (Fig. 2C, D). Seedlings treated with 100 nM NAA plus 100 μM 5-BDPU showed the highest induction of GUS activity, as GUS staining was clearly visible beyond the root-hypocotyl junction (see the asterisk in Fig. 2D). The effect of 6-BDPU was not tested in these experimental conditions as it was assumed that it could be similar to that showed by 5-BDPU.

Effect of BDPUs on adventitious rooting of apple stem slices

The apple stem slice test is an endogenous growth regulators-free system that has been developed for studying the adventitious root formation in woody species performing a rapid and reproducible rooting response highly medium-dependent (Van der Krieken et al. 1993). Adventitious rooting enhancement of apple stem slices by BDPUs in the presence of IBA has been previously described (Ricci et al. 2006). When 1 or 2 μM 5-BDPU were supplemented to the culture medium in the simultaneous presence of 1 μM NAA, the percentage of rooting increased, reaching a maximum at 2 μM (48.6 and 62.0 % over the control, respectively). Yet, the mean root number obtained per rooted slice in the presence of each mixture did not differ significantly from that obtained in the presence of 1 μM NAA alone (data not shown). The percentage of rooting was significantly decreased (10.5 % over the control at 4 μM) at higher BDPU concentrations (Fig. 3). An emerging presence of callus and no roots were observed in each slice in the presence of the highest 5-BDPU concentration (8 μM). The effect of 6-BDPU was not tested in these experimental conditions as it was assumed that it could be similar to that showed by 5-BDPU.

Effect of BDPUs on adventitious rooting of Pinus cuttings

The effect of different concentrations of 5-BDPU or 6-BDPU on the adventitious rooting of hypocotyl cuttings from 21-day-old *P. radiata* seedlings was analyzed in the absence or in the presence of different concentrations of IBA or NAA.

No roots or toxic effects were observed when the cuttings were cultured in distilled water, DMSO solution or in the presence of the different BDPU concentrations without exogenous auxin supplementation (data not shown).

Table 2A shows the effect of 5-BDPU or 6-BDPU plus IBA on the percentage of rooted hypocotyl cuttings. The percentages of rooted hypocotyl cuttings increased in the presence of 1 or 10 μM 5-BDPU plus 0.1 μM IBA if compared with the percentage of rooting quantified when the same concentration of IBA was exogenously applied. However, the increase of rooting was not statistically significant. 6-BDPU was ineffective in the presence of 0.1 μM IBA. The combinations of 10 μM 5-BDPU or 6-BDPU plus 1 μM IBA enhanced the adventitious root formation as the percentages of rooted cuttings were significantly higher (Kolmogorov–Smirnov test, $p < 0.1$) than that of the same IBA concentration alone (73.3 and 60.0 % respectively vs. 33.3 %). No callus formation was observed at the base of the cuttings. In the presence of 0.1, 1 or 10 μM 5-BDPU or 6-BDPU plus 10 μM IBA the percentages of rooted hypocotyl cuttings did not differ from the control (10 μM IBA). Callus induction was observed when cuttings were cultured in these culture conditions. The mean root number per rooted cutting did not significantly differ from that obtained in the presence of exogenous IBA (data not shown) in all combinations tested.

Table 2B shows the effect of the different NAA concentrations and the effect of 10 μM 5-BDPU or 6-BDPU plus 0.1 μM NAA on the percentage of rooted hypocotyl cuttings.

The percentage of rooted cuttings increased as the NAA concentration increases (66.7 and 100 % respectively) in the presence of 0.1 and 1 μM NAA alone. A large-sized

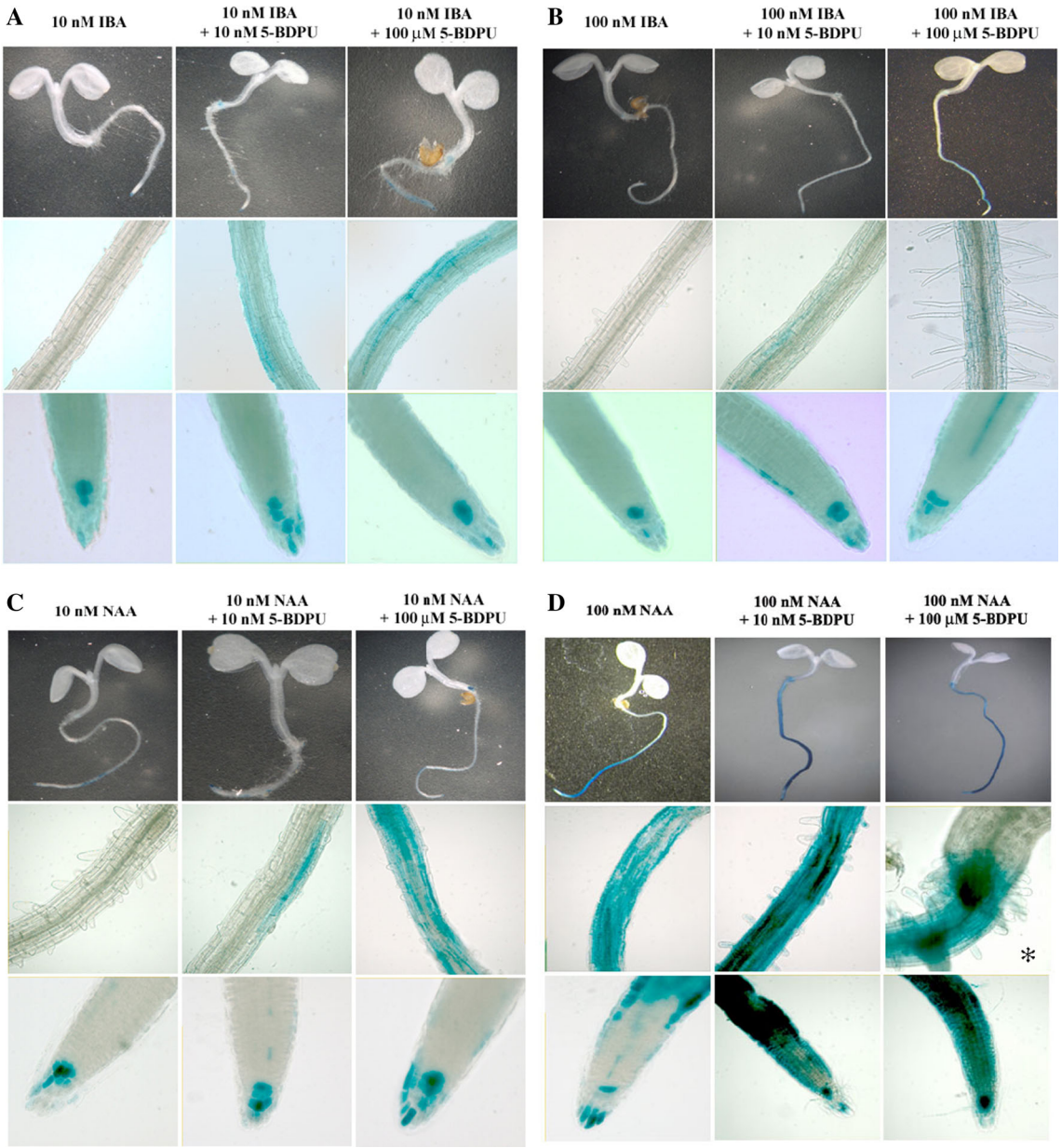


Fig. 2 Histochemical localization of GUS activity. **A–D** show the effects of the different treatments on *DR5::GUS* expression in whole seedling, part of primary root, root tip (from the *top* to the *bottom* of the panels). *Asterisk* indicates the only condition in which staining goes beyond the transition zone between root and hypocotyl. Experiments were repeated three times and representative phenotypes are shown (n = 30)

callus was induced in the presence of 10 µM NAA. Adventitious roots could not be identified in such mass of callus.

The combination of 10 µM 5-BDPU plus 0.1 µM NAA enhanced the adventitious root formation as the percentage of rooted cuttings was significantly higher (Kolmogorov–Smirnov test, $p < 0.1$) than that obtained in the control (0.1 µM NAA) (84.0 vs. 66.7 %). The percentage of rooted cuttings was reduced (57.3 vs. 66.7 %) in the presence of 10 µM 6-BDPU plus 0.1 µM NAA. Nevertheless, no callus formation was observed at the adventitious root emergence sites in both the tested mixtures. The mean root number per rooted cutting did not differ significantly from that obtained in the controls in the presence of each aforementioned combination (data not shown).

A histological time course of adventitious rooting was also carried out to evaluate the cellular events leading to the formation of adventitious roots in the presence of 5-BDPU and IBA. 5-BDPU was selected based on the effectiveness, if compared with 6-BDPU, in all combinations tested. Results are shown in Fig. 4. As reported, hypocotyls from 21-day-old *P. radiata* seedlings showed a

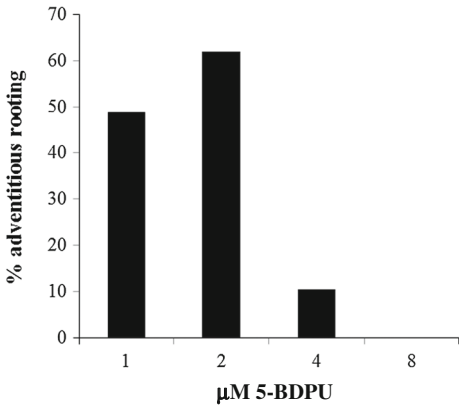


Fig. 3 Adventitious rooting of apple stem slices. Effect of different concentrations of 5-BDPU on adventitious rooting of apple slices in the simultaneous presence of 1 µM NAA. The number of rooted slices was counted after 14 days. The results are expressed as a percentage of the control (1 µM NAA alone) by the formula [(T-C/C) × 100]

primary structure, with 5–6 poles of primary xylem and phloem marked by a centrifugal resin canal (time 0, for comparison see Ricci et al. 2008). When cuttings were cultured in the absence of exogenous auxin, the presence of globular-shaped groups of dividing cells, was observed after 10 days of culture.

These clusters of cells are closely localized to the resin canals, at the same position adventitious roots are formed (Ricci et al. 2008). The clusters subsequently increased their capacity of divisions; however, no root primordia were visible (Fig. 4**A**–**C**). Divisions preceeding the morphological organization of the root meristem were observed in the vascular parenchyma after 6 days when cuttings were cultured in the presence of 1 µM IBA. Root primordia were not visible before 13 days (Fig. 4**D**–**F**). When cuttings were cultured in the presence of 10 µM 5-BDPU, the results were similar to that obtained in the absence of exougenous auxin. However, globular-shaped groups of dividing cells were still clearly visible after 13 days of culture (Fig. 4**G**–**I**). When cuttings were cultured in the presence of 10 µM 5-BDPU plus 1 µM IBA, the clusters of cells located centrifugal to the resin canals were visible after 6 days of culture, and cell divisions reorienting into a new plane organizing some root primordia were clearly visible after 10 days of culture. Root growth was observed after 13 days (Fig. 4**J**–**L**).

Effect of BDPUs on auxin distribution and auxin-responsive genes

Endogenous distribution of IAA in transverse sections of hypocotyl cuttings in the presence of BDPUs was analyzed by IAA immunolocalization at 1, 6 and 10 days during the adventitious root formation. 5-BDPU in the presence of

Table 2 Adventitious rooting of Pinus cuttings

(µM)	0.1 µM IBA plus			
	0	0.1	1	10
(A)				
5-BDPU	0.0	6.6	13.3	20.0
6-BDPU	0.0	0.0	0.0	0.0
(µM)	1 µM IBA plus			
	0	0.1	1	10
5-BDPU	33.3	26.6	33.3	73.3*
6-BDPU	33.3	26.6	40.0	60.0*
(µM)	10 µM IBA plus			
	0	0.1	1	10
5-BDPU	93.3	93.3	93.3	100.0
6-BDPU	93.3	100.0	100.0	100.0

(µM) NAA	0	0.1	1	10
(B)				
	0.0	66.7	100.0	n.d.
10 µM 5-BDPU		84.0*		
10 µM 6-BDPU		57.3		

n.d. not detectable

(A) Effect of different concentrations of 5-BDPU or 6-BDPU on adventitious rooting of *P. radiata* hypocotyl cuttings in the simultaneous presence of different concentrations of IBA. Results are expressed as percentage of rooted cuttings (* significantly different, Kolmogorov–Smirnov test, $p < 0.1$). For each treatment 15 hypocotyls were used and the experiments were repeated twice

(B) Effect of different concentrations of NAA and effect of 10 µM 5-BDPU or 6-BDPU plus 0.1 µM NAA on adventitious rooting of *P. radiata* hypocotyl cuttings. Results are expressed as percentage of rooted cuttings (* significantly different, Kolmogorov–Smirnov test, $p < 0.1$). For each treatment 15 hypocotyls were used and the experiments were repeated twice

exogenous auxin affects endogenous auxin localization at specific sites mostly at 6 days of culture (Fig. 5). Localized IAA signals were observed in discrete globular-shaped structures in the hypocotyl cuttings treated with 10 µM 5-BDPU and 1 µM IBA (Fig. 5**G**–**I**). No localized signal was observed in the controls, in the absence of auxin (data not shown), in the presence of 1 µM IBA (Fig. 5**A**–**C**) or in the only presence of 5-BDPU (Fig. 5**D**–**F**) at this time point. No major changes in auxin distribution were observed at 1 and 10 days of adventitious root formation in the presence of BDPUs (data not shown). A similar pattern of endogenous distribution of IAA was observed when cuttings were cultured in the presence of 1 µM 2,3-MDPU or 1 µM 2,3-MDPU plus 1 µM IBA at the same time course (Fig. 5**J**–**L** and **M**-**O** respectively).

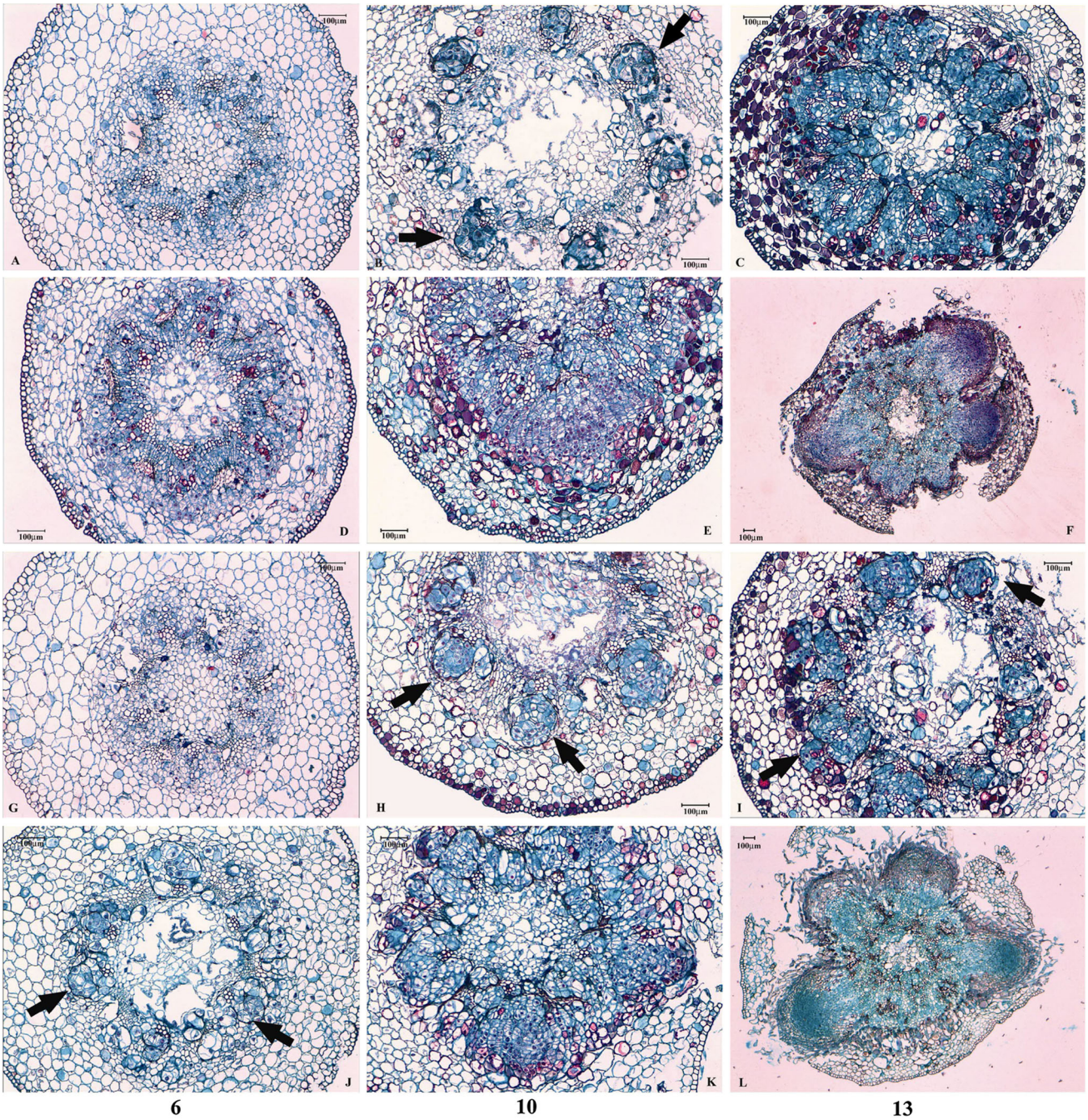


Fig. 4 Histological analysis of adventitious rooting of Pinus cuttings. Cross sections of the base of 21-day-old hypocotyl cuttings cultured in different culture conditions: **A**–**C** in the presence of water at day 6, 10 and 13; **D**–**F** in the presence of 1 μM IBA at day 6, 10 and 13; **G**–**I** in the presence of 10 μM 5-BDPU at day 6, 10 and 13; **J**–**L** in the presence of 10 μM 5-BDPU plus 1 μM IBA at day 6, 10 and 13. *Arrows* indicate some examples of clusters of cells

In order to approach the mode of action of BDPUs and their possible interaction with the auxin signalling pathways, the expression of two genes, a *P. radiata SCARECROW-LIKE1* gene (*PrSCL1*) and a *P. radiata SHORTROOT* gene (*PrSHR*), was analyzed in the presence of 1 μM IBA plus 10 μM 5-BDPU or 6-BDPU-treated hypocotyl cuttings from 21-day-old seedlings during the initial 24 h of the root induction process (Sanchez et al. 2007; Ricci et al. 2008; Solé et al. 2008). Maximum mRNA levels of both genes had also been measured at this

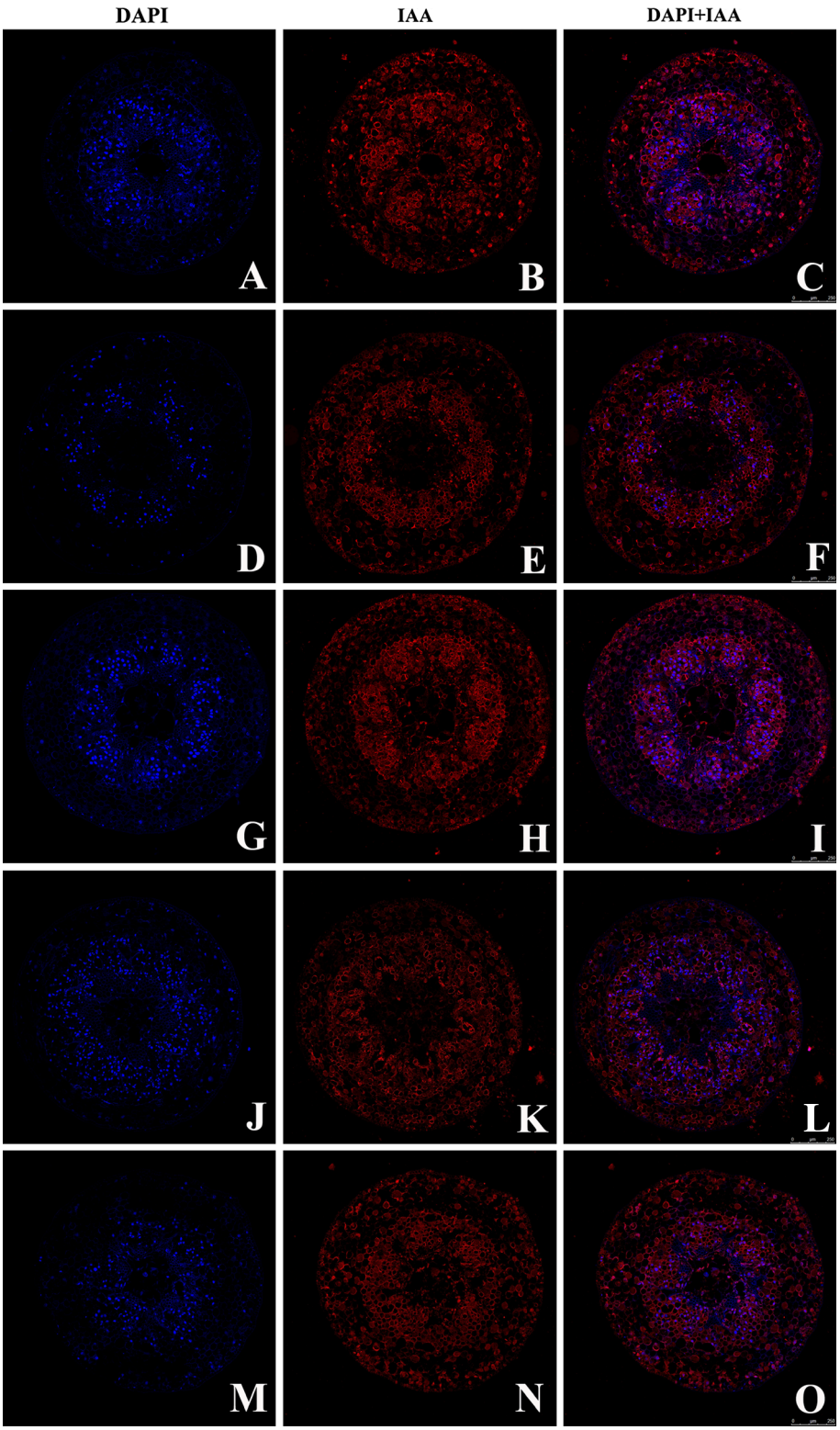


Fig. 5 Endogenous distribution of IAA in transverse sections of the base of 21-day-old Pinus hypocotyl cuttings at 6 days of culture. Different culture conditions: **A–C** in the presence of 1 μM IBA; **D–F** in the presence of 10 μM 5-BDPU; **G–I** in the presence of 10 μM 5-BDPU plus 1 μM IBA; **J–L** in the presence of 1 μM 2,3-MDPU; **M–O** in the presence of 1 μM 2,3-MDPU plus 1 μM IBA

stage (Sanchez et al. 2007; Ricci et al. 2008; Solé et al. 2008). Non-treated cuttings and cuttings exposed to 1 μM IBA, 10 μM IBA, 10 μM 5-BDPU and 6-BDPU alone were used as controls. Results are expressed as relative values to time 0. Both 5-BDPU and 6-BDPU increase *PrSCL1* mRNA levels in the presence of IBA (Fig. 6A), the level of transcripts being significantly higher ($p < 0.01$) than the ones measured in the presence of auxin alone at

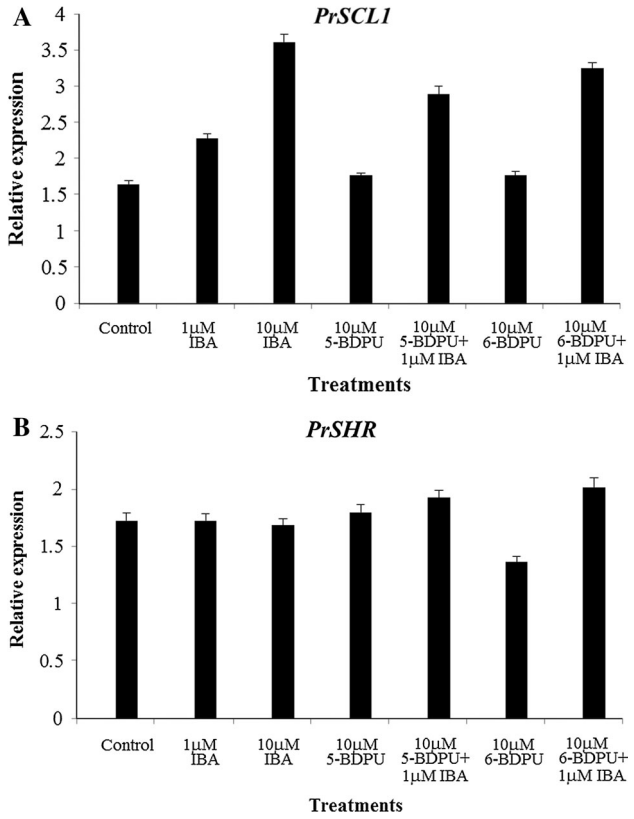


Fig. 6 Expression of *PrSCL1* gene (**A**) and *PrSHR* gene (**B**) in the presence of different concentrations of auxin and BDPUs during adventitious root formation. qRTPCR was performed using RNA extracted from the base of hypocotyl cuttings in the presence of IBA or IBA plus BDPUs as indicated. Results are expressed as mean values of relative expression to time 0 ± SE from two biological replicates. The increase of *PrSCL1* gene expression within the first 24 h in the presence of 10 μM IBA and 1 μM IBA plus 10 μM BDPUs was significant at $p < 0.01$. Control: untreated cuttings

the same concentration. The expression of a *P. radiata SHORT-ROOT* gene (*PrSHR*) was not significantly affected in the presence of both BDPUs alone or combined with auxin (Fig. 6B).

Discussion

The formation of adventitious roots is a crucial step for either in vivo or in vitro large-scale vegetative propagation. It is the bottleneck in breeding programs and to maintain superior genotypes. In addition, adventitious rooting is a complex physiological process, affected by several endogenous and exogenous factors, which has been extensively investigated but not fully understood. Auxin plays a pivotal role in stimulating adventitious root formation. However, the type of auxin, concentration or exposure time could affect the process. Many efforts have been made to enhance adventitious rooting by the combination of auxin with other compounds that could affect the competence of cells to form adventitious roots. Starting from the study previously carried out with MDPUs and from the knowledge obtained (Ricci et al. 2001, 2003, 2008), we planned a multi-faced set of experiments to better analyze the biological activity of 1,3-di(benzo[*d*]oxazol-5-yl)urea (5-BDPU) and 1,3-di(benzo[*d*]oxazol-6-yl)urea (6-BDPU), two symmetrical urea derivatives structurally related to MDPUs, that have been already described as adventitious rooting adjuvants (Ricci et al. 2006).

Adventitious rooting enhancement by BDPUs is conserved in distantly-related species and could be related to auxin distribution and sensitivity of cells to respond to auxin

The effect of BDPUs on the rooting capacity was performed by treatments using different BDPU concentrations alone or in combination with different exogenous auxins, such as IBA and NAA, in herbaceous and woody plants using different experimental systems, such as etiolated seedlings of the model plant *A. thaliana*, apple stem slices and *P. radiata* hypocotyl cuttings.

BDPUs are ineffective when supplemented to the hypocotyl cuttings from 21-day-old *P. radiata* seedlings in the absence of exogenous auxin (data not shown), whereas they enhanced the adventitious root formation (Table 1A) when supplemented to the *A. thaliana* etiolated seedlings at specific concentrations. The BDPUs could interact with the endogenous auxin pool triggering adventitious root formation (Diaz-Sala et al. 2002) in the *A. thaliana* etiolated seedlings since adventitious roots are formed in the control condition as well, while exogenous auxin is required for adventitious root formation in *P. radiata* hypocotyl cuttings (Diaz-Sala et al. 1996).

The enhancement of adventitious rooting in the presence of BDPUs and exogenous auxin depends on the type of auxin. In the simultaneous presence of IBA, a weaker auxin than NAA, the rooting enhancement is obtained in the presence of high BDPU concentrations, both in the *A. thaliana* etiolated seedlings (Table 1B) and in the *P. radiata* hypocotyl cuttings (Table 2A), confirming the results already reported using the apple stem slice test (Ricci et al. 2006). The histological time course of adventitious rooting in *P. radiata* hypocotyl cuttings gives clear information about the process. After sectioning the *P. radiata* hypocotyl cuttings cultured in different treatment conditions, it was extremely evident that the globular-shaped clusters of cells, visible after 10 days of culture either in the absence of exogenous auxin (Fig. 4B) or in the presence of 10 μM 5-BDPU alone (Fig. 4H), did not evolve into root primordia, confirming that BDPUs do not show any "rooting activity" per se, as already reported by Ricci et al. (2006).

However, these clusters were still clearly visible after 13 days of treatment in the presence of 5-BDPU (Fig. 4I), suggesting that these urea derivatives could interact with the sensitivity of cells to respond to auxin stimulus promoting the localization of cell divisions without callus formation.

The clusters of cells are induced after 6 days of culture (Fig. 4J), root primordia and roots are visible after 10 and 13 days of culture, respectively (Fig. 4K, L) in the presence of 10 μM 5-BDPU plus 1 μM IBA. It seems that the adventitious rooting process is not only enhanced but also induced in advance, confirming the BDPU adjuvant activity. Cell divisions are mostly localized in globular-shaped areas located centrifugal to the resin canal, and this could explain the absence of callus formation at the base of the cuttings. However, in the simultaneous presence of NAA, a stronger auxin than IBA, the rooting enhancement is obtained in the presence of low BDPU concentrations in both the *A. thaliana* etiolated seedlings (Table 1C) and the apple stem slices (Fig. 3). In the latter experimental system, the rooting enhancement dramatically decreases with the increasing BDPU concentrations, thus, it seems that explants undergo the effect of the presence of supraoptimal auxin concentration, which is also shown by the induction of a significant amount of callus. Furthermore, the adventitious rooting enhancement is only observed in the simultaneous presence of the lowest NAA concentration (0.1 μM, tenfold lower than that of IBA, Table 2B) and 10 μM 5-BDPU in the *P. radiata* hypocotyl cuttings. Enhancement of rooting could not be quantified in the presence of higher NAA concentrations, neither as percentage of rooting nor as number of induced roots, since a high amount of callus was induced and the outgrowth of the root primordia could be inhibited (De Klerk et al. 1997). The effect of BDPUs on the localized cell division at the root initiation sites, the formation of the clusters of cells during the early stages of adventitious root formation (6 days) in the presence of low concentration of exogenous auxin and the inverse relationship between rooting capacity and concentrations of BDPUs and NAA, a stronger auxin than IBA, suggest that BDPUs could affect rooting capacity promoting the localization or preventing the dispersion of auxin, increasing the sensitivity of cells to the auxin stimulus, or both.

BDPUs modify auxin responsiveness in the presence of exogenous auxin in *DR5::GUS* transgenic plants

DR5 has been used in many studies to evaluate *in planta* specific sites and patterns of auxin distribution and response, as its activity is thought to reflect endogenous auxin levels in different plant organs, tissues or cells (Sabatini et al. 1999; Casimiro et al. 2001; Benková et al. 2003; Bai and DeMason 2008). Even if an empirical auxin quantification dependent on histochemical GUS staining has been proposed (Pozhvanov and Medvedev 2008), it is widely accepted that the qualitative information usually obtained by the visualization of staining intensity or extent and by the appearance of new sites of staining is related to the different treatments to which seedlings have been subjected. Accordingly to already reported data (Bai and DeMason 2008), we demonstrate that, compared to the IBA treatments, NAA induces very high GUS expression, either because it enters cells more efficiently or because of different metabolism. At the same time, it is clear that in the presence of the same type of auxin at the same concentration, the supplementation of each 5-BDPU concentration causes a magnification of the GUS staining, as a higher intensity and extension of the stain is shown. As *DR5* is generally thought to be sensitive to auxin in a dosage-dependent manner, we could speculate that 5-BDPU affects auxin influx or auxin transport along the seedlings, enhances cell sensitivity to auxin, or both. Whatever the mode of action, it is unrelated to the type of auxin used in these experimental conditions, while the effect is strictly related to the auxin strength, as it is weaker in the presence of IBA than in the presence of NAA.

BDPUs modify spatial auxin distribution and the mRNA levels of genes induced in the presence of exogenous auxin in pine cuttings during adventitious rooting

BDPUs influence auxin localization at specific sites during adventitious root formation in pine, specifically at 6 days of the root initiation process. The auxin localization at specific discrete globular-shaped clusters of cells could be due to the capacity of 5-BDPUs to interact with auxin transport and to originate auxin maxima at specific sites of dividing cells or to the capacity to organize root meristems accumulating auxin faster. BDPUs could directly affect distribution of auxin facilitating the transport of both the endogenous IAA or the exogenous IBA, either as IBA or converted into IAA (Bartel et al. 2001), or could cooperate with auxin to indirectly favour their own transport at specific locations (Nick et al. 2009) allowing root meristem organization in the presence of low exogenous auxin.

This could also explain the inhibition of the rooting response obtained in the presence of high auxin concentrations, especially when a stronger auxin, such as NAA, is used. Accumulation of auxin characterize the early derivatives of the adventitious root founder cells in in vitro cultured thin-cell layers from *Arabidopsis* (Della Rovere et al. 2013).

However, the effect of BDPUs on the expression of two genes induced at the very early stages of adventitious

rooting shows that the mRNA levels of *PrSCL1* are high in the presence of BDPUs when combined with low auxin concentrations after 24 h of culture. On the other hand, the presence of BDPUs does not affect the expression of *PrSHR*. *PrSCL1* is in the auxin-dependent signaling pathway resulting in the adventitious root formation, and *PrSCL1* mRNA levels are increased in the presence of high auxin concentrations (Sanchez et al. 2007). On the contrary, *PrSHR* does not respond to the presence of exogenous auxin in this system (Solé et al. 2008). The presence of BDPUs seems to enhance the effect of low auxin concentrations on the expression of *PrSCL1*, but BDPUs do not significantly affect the expression of *PrSHR*. This result could indicate that the effect of BDPUs could be executed via the auxin-signalling pathway before the activation of cell divisions, and before detectable changes of auxin distribution that originate the root primordia. Similar results were obtained when cuttings were treated with 2,3-MDPU (Ricci et al. 2008).

The data presented in this manuscript demonstrate that

- the adventitious rooting adjuvant activity shown by BDPUs is conserved in distantly-related herbaceous and woody species, regardless the type of exogenous auxin, IBA or NAA, sometimes added;
- the differences in optimal BDPU concentrations are likely the consequence of differences in the strength of the exogenous auxins, IBA and NAA, i.e. the stronger the auxin, the lower is the BDPU concentration required to enhance the formation of adventitious roots emerging directly without any callus mass, forming a good quality root system;
- BDPUs probably interact with auxin-signaling pathways and with auxin distribution before and after the cell divisions that originates the adventitious root meristem.

The results support the hypothesis that MDPUs and BDPUs, having similar electronic structure as well as similar chemical–physical requirements, constitute a category of adventitious rooting adjuvants.In broad terms, from our data we could speculate that these urea derivatives act as auxin adjuvants, the adventitious rooting being only one of the physiological effects in which auxins are involved. Thus, further investigations will be focused on the influence that BDPUs and/or MDPUs could exert on other auxin-driven processes, like for example tropisms, cell elongation, flower maturation, vascular tissue differentiation.

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