UNIVERSITA' DEGLI STUDI DI PARMA
Dottorato di ricerca in Biologia Vegetale
Ciclo XXV

Insights into adjuvant activity of BDPUs

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2014

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List of publications

The present thesis is based on the following papers:

Brunoni F., Rolli E., Dramis L., Incerti M., Abarca D., Pizarro A., Díaz-Sala C., and Ricci A.

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

Manuscript under review submitted to Plant Cell, Tissue and Organ Culture (2013)

Brunoni F., Rolli E., Incerti M., Ruffoni B., and Ricci A.

Effect of urea derivatives on the in vitro adventitious rooting of strawberry tree microcuttings

Acta Italus Hortus, 6:53-62 (2012)

Introduction

Plant growth and development are regulated by a structurally unrelated collection of small molecules called plant hormones. Plant hormones integrate diverse environmental clues with a plant's genetic program (Santner and Estelle, 2009). The terms 'plant hormones' or 'phytohormones' have been adapted to plants from the mammalian concept of hormone. Plant hormones, similarly to the mammalian ones, were originally thought to produce growth responses at distance from their site of synthesis and control physiological responses in target tissue via their concentration. But this concept has changed dramatically with the continuing discoveries of hormone action and metabolism in plants. It is now known that the synthesis of all plant hormones, as a rule, can occur in any type of living cell, even if certain tissues are privileged sites of synthesis and export for hormone types. They can also act in the tissue or even within the cell in which they are synthesized. Importantly, phytohormone control is not achieved only by concentration, but also by changes in sensitivity of the cells to the compounds. In line with these findings, it has been proposed to replace the terms 'plant hormones' or 'phytohormones' with 'plant growth regulators' (PGR). However, this term is still imperfect because it does not cover the control of developmental processes in addition to growth. Moreover, it has progressively included synthetic 'plant growth regulators' and may not be used in reference to endogenous regulators. Thus, 'plant hormones' or 'phytohormones' remain the most appropriate terms to define the 'plant chemical messengers' (Gaspar *et al.*, 2003). The 'classical' phytohormones, identified during the first half of the twentieth century, are auxin, abscisic acid, cytokinin, gibberellins and ethylene. More recently, several additional compounds have been recognized as hormones, including bassinosteroids, jasmonate, salicyclic acid, nitric oxide and strigolactones (Santner and Estelle, 2009). Among phytohormones, auxins, cytokinins and auxin-cytokinin interactions are usually considered to be the most important for regulating growth and organized development in plant tissue and organ cultures, as these two classes of phytohormones are generally required (Gaspar *et al.*, 1996). Since the discovery of auxin/cytokinin responses in plant cell culture systems, many additional compounds have been

discovered that influence growth and morphogenesis. New bioactive compounds can be obtained by structural modification of bioactive natural products, by available information about the quantitative analysis of chemical structure-biological activity relationships of different series of compounds, or by bioisosteric transformations. The newly synthesized compounds involve important deviations from the structure of initial compounds. These modifications have some advantages. First, synthetic compounds are metabolically stable because the chemical modifications make them less susceptible to degradative enzymes (*i.e.* oxydases). Second, they manifest a large scale of physiological activities. Third, they are biologically active at lower concentrations. Fourth, they have an enhanced duration of biological action. Last but not least, they are considerably less expensive (Yonova, 2010).

Auxins

Auxins are intimately involved in such processes as cell growth expansion through cell wall acidification, initiation of cell division, meristem maintenance, establishment of cellular patterning and promotion of vascular differentiation (reviewed in Perrot-Rechenmann, 2010). In organized tissue, auxins are the key players in maintaining apical dominance affecting abscission, promoting root formation, tropism curvatures, delaying leaf senescence and fruit ripening (reviewed in Gaspar *et al.*, 1996). By far the most prominent and best-studied auxin is indole-3-acetic acid (IAA; **fig. 1a**), which has been linked to such diverse processes. The main organogenic effect observed following external application of IAA was rooting (reviewed in Kevers *et al.*, 2009). Concurrent with the IAA discovery as a plant growth hormone, a new auxin was synthesized: indole-3-butyric acid (IBA; **fig. 1b**) also promoted rooting (reviewed in Ludwig-Müller, 2009). IBA and IAA are nearly identical, but IBA carries a four-carbon side chain, whereas IAA carries a two-carbon side chain (Strader and Bartel, 2011). IBA has been identified as a natural product in many species. In *Arabidopsis* seedlings, IBA represents 25-30% of total auxin (reviewed in Ludwig-Müller, 2000). Two main types of synthetic plant growth regulators with auxin-like activity, including root elongation inhibition and lateral root promotion, have been described: 1-naphtalene acetic acid (NAA; **fig. 1c**) and 2,4-

dichlorophenoxyacetic acid (2,4-D; **fig. 1d**). 2,4-D is often used for embryogenic callus induction and suspension cultures, whereas NAA is able to lead organogenesis (Gaspar *et al.*, 1996).

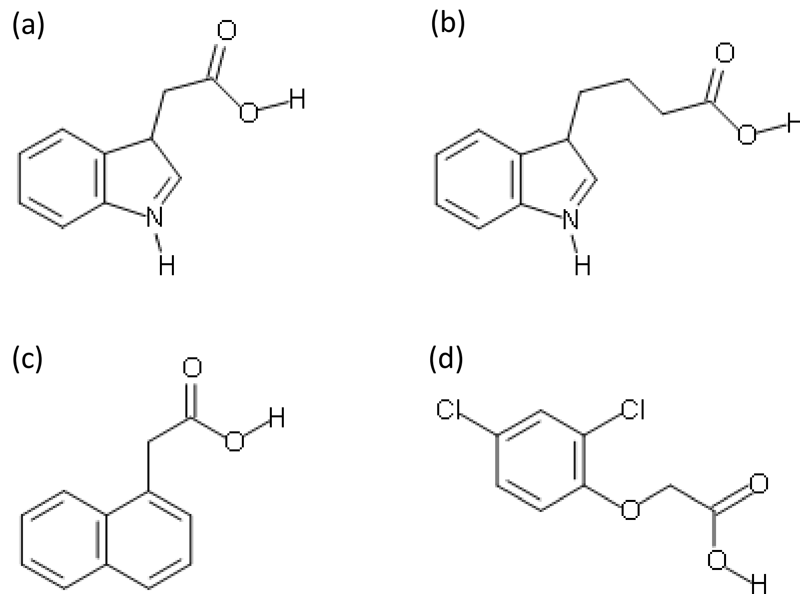

Figure 1
Chemical structure of some naturally occurring auxins and auxin-like regulators. (a) Indole-3-acetic acid (IAA) is the most commonly detected natural auxin. (b) Indole-3-butyric acid (IBA) has been indentified as natural auxins, as well. (c) 1-naphtalene acetic acid (NAA) and (d) 2,4-dichlorophenoxyacetic acid (2,4-D) are synthetically prepared auxins and often are more effective than the natural ones.

Since auxins influence virtually every aspect of plant growth and development, a fast and tight control of auxin levels is necessary. Such control is realized via two basic processes: auxin metabolism (biosynthesis, conjugation, de-conjugation and degradation), and its transport within and among cells (Hošek *et al.*, 2012).

Auxin metabolism

IAA biosynthesis is non-cell autonomous since it is locally produced mostly in the aerial parts of the plant *e.g.* young developing leaves and shoot apical meristem. In addition, the entire root also has IAA synthesis capacity and the synthesis rates are particularly high in the primary root tip and in the tips of developing lateral roots, indicating that the root

system might represent a source of IAA (Ljung *et al.*, 2005). Auxin local biosynthesis contributes to the generation and maintenance of local auxin gradients, resulting in a temporal and spatial regulation. Several pathways contribute to *de novo* IAA production and its biosynthesis is complex. IAA can be biosynthesized from tryptophan, indole or indole-3-glycerol phosphate (reviewed in Woodward and Bartel, 2005; Zhao, 2010; Mashiguchi *et al.*, 2011). The side chain of pre-formed IAA can be reversibly converted into IBA by peroxisomal enzymes (Woodward and Bartel, 2005; Strader and Bartel, 2011). Hence, the peroxisomal β-oxidation of endogenous IBA can also supply plants with IAA (Bartel *et al.*, 2001). IAA can be, then, inactivated through conjugation and oxidation (reviewed in Woodward and Bartel, 2005). Mutant analyses have identified some of the enzymes and components of the pathways that regulate auxin metabolism (Boerjan *et al.*, 1995; Seo *et al.*, 1998; Delarue *et al.*, 1998; Barlier *et al.*, 2000; Mikkelsen *et al.*, 2004; Zhao, 2010). IAA can be conjugated, usually with amino acids, sugars or inositol (Nordström *et al.*, 1991; Woodward and Bartel, 2005; Ludwig-Müller, 2009). IAA catabolism involves oxidative decarboxylation by peroxidases or alternative non-decarboxylating pathways (Fogaça and Fett-Neto, 2005). Most conjugates are resistant to oxidative enzymes and can revert to free auxin, creating a useful mode of regulation of auxin activity. Furthermore, proposed functions for these conjugates include storage, transport, compartmentalization and excess IAA detoxification (Woodward and Bartel, 2005).

Auxin transport

Once biosynthesized, auxin is, then, generally transported by two distinct pathways. First, auxin is translocated rapidly from the source tissues by the bulk flow of other metabolites in mature phloem. Second, auxin is transported by a much slower, carrier-dependent, cell-to-cell polar transport (polar auxin transport, PAT), from the shoot apex towards the root apex and also in different tissues (Tanaka *et al.*, 2006; Petrášek and Friml, 2009). In shoots, auxin moves unidirectionally from the apex to the root (basipetal transport). In roots, auxin transport is more complex, with two distinct polarities. Auxin moves acropetally (towards the root apex) through the central cylinder and basipetally (from the root apex towards the base) through the outer layers of root cells (reviewed in Muday and DeLong, 2001). The physical-chemical nature of auxin molecules underlies the

mechanism of their transport across the plasma membrane. Since auxin molecules are weak acids, the degree of their dissociation depends on pH value (Hošek *et al.*, 2012). Auxin can enter into cells by passive diffusion or by active transport. Undissociated IAA molecules enter cells preferentially by passive diffusion across plasma membrane. Since intracellular pH is slightly alkaline (7.4-7.5) and not acid (5.5) as in the extracellular space, auxin molecules are trapped in the cellular cytoplasm in a complete dissociated form (IAA^-). Several types of plasma membrane-located facilitators, localized asymmetrically on the plasma membrane, actively transport auxin anions (Woodward and Bartel, 2005; Hošek *et al.*, 2012). IAA^- influx into cells is mediated by $2H^+$ cotransporters of the AUXIN1/LIKE-AUX1 (AUX1/LAX) family (Hošek *et al.*, 2012). Instead, the efflux of IAA^- is specifically regulated by transmembrane proteins belonging to the PIN-FORMED (PIN) protein family. Some orthologs to the mammalian ATP-binding cassette subfamily B (ABCB)-type transporters of the MULTIDRUG RESISTANCE-like/phosphoglycoprotein (ABCB/MDR/PGP) proteins cowork with PIN proteins to regulate IAA^- efflux (Woodward and Bartel, 2005; Petrášek and Friml, 2009). The plasma-membrane PIN proteins often display specific polar localization to a particular cellular face, determining the direction of IAA flow through cells and tissues. In contrast, ABCB/MDR/PGP proteins are more symmetrically localized to the plasma membrane than PIN proteins, suggesting that they can facilitate non-polar IAA efflux that contributes to long-distance auxin transport, potentially delivering IAA to PIN proteins (reviewed in Strader and Bartel, 2011). Despite the chemical similarity of IAA and IBA, distinct carrier proteins facilitate IBA and IAA movement (Tanaka *et al.*, 2006; Strader and Bartel, 2011). It is interesting that the synthetic analogue, NAA, while being an excellent substrate for cellular efflux, is only poorly actively transported by influx. The lack of influx is compensated by the passive diffusion of NAA into cells due to its lipophilic nature (Tanaka *et al.*, 2006). However, NAA is rapidly metabolized to NAA glucosyl ester. This metabolite then remains trapped inside cells because it is not substrate for the auxin efflux transporters (Hošek *et al.*, 2012). Another synthetic auxin, 2,4-D, is transported by influx, but only poorly by efflux, resulting in its retention within cells (Tanaka *et al.*, 2006).

Auxin signalling

Auxin perception occurs at two receptors, transport inhibitor response 1/auxin signalling F-box 1-3 (TIR1/AFB 1-3) and auxin-binding-protein 1 (ABP1). TIR1/AFB F-box proteins function as auxin nuclear receptors and are an integral part of the SKP1-CULLIN-F-BOX PROTEIN (SCF). $SCF^{TIR1/AFB}$ is an ubiquitin-ligase complex which mediates ubiquitinylation of repressors of auxin signalling, such as auxin/indole-3-acetic acid (Aux/IAA). Once ubiquitinylated, these substrates are targeted to the 26S proteasome for degradation (Quint and Gray, 2006). The auxin molecule acts as 'molecular glue' between TIR1 and Aux/IAA, binding both proteins. Once the TIR1-auxin-Aux/IAA complex is formed, the hormone is trapped within the auxin cavity, presumably until the ubiquitinated protein is released (Mockaitis and Estelle, 2008). Another protein, ABP1, has been shown to participate in auxin responses at the plasma membrane, to mediate auxin control of cell division and cell elongation, and lately, to modulate regulation of early auxin response genes supporting its presumed function as an extracellular receptor of auxin (Tromas and Perrot-Rechenmann, 2010). However, the contribution of ABP1 as an auxin receptor is still matter of debate. Once auxin enters a cell it is capable of modulating the transcription of a large number of genes. The most intensively characterized auxin-response cascade involes proteins transcribed by three genes: *SMALL AUXIN-UP RNAs* (*SAURs*), *GH3*s and *Aux/IAA* inducible genes (Li *et al.*, 2009; Tromas and Perrot-Rechenmann, 2010). *SAURs* genes code for highly conserved, short-lived, small transcripts. Although their function is not clearly established, they have been proposed to act as calmodulin-binding proteins. *GH3* genes code for conjugating enzymes, a class of which acts as feedback regulators by reducing free auxin levels (Tromas and Perrot-Rechenmann, 2010). Aux/IAAs are short-lived proteins and localize to the nucleus. Four conserved motifs are found in most Aux/IAA proteins, and these are referred to as domains I, II, III and IV. Domain II plays a role in destabilizing Aux/IAA proteins, and may be a target for ubiquitination. Domain III is part of a motif which plays a role in dimerization/multimerization of Aux/IAA proteins and heteromerization between Aux/IAA and Auxin response factor (ARF) proteins (Hagen and Guilfoyle, 2002). Most ARFs possess a DNA-binding domain at the N-terminal and are transcription factors involved in the regulation of early auxin-response genes. In general they act as activators if they contain a glutamine/serine/leucine-rich (QSL-rich) middle region or as repressors if they

contain a serine or serine/proline/glycine-rich middle domain. A number of ARFs exhibit two C-terminal protein-protein interaction domains referred as domains III and IV. These domains III and IV are shared by the Aux/IAAs and may trigger homo- or hetero-dimerization with ARFs or Aux/IAA repressors. In the absence of auxin or at low auxin levels, ARF and Aux/IAA heterodimers might be formed. The N-terminal protein-protein interaction domain of the Aux/IAAs can also interact with other transcriptional repressors to exert repression, resulting in inhibition of the transcription of auxin response genes (Tromas and Perrot-Rechenmann, 2010). At higher auxin levels, auxin interacts with TIR1-AFB1-3 receptors of the SCF$^{TIR1/AFB}$ complex and Aux/IAA repressors, resulting in Aux/IAA ubiquitylation and degradation by 26S proteosome. This event activates ARFs, which in turn initiate transcription of early auxin-induced genes (Niklas and Kutschera, 2012; Vanneste and Friml, 2009). Because most early auxin-induced gene products do not carry out direct physiological reactions but rather regulate transcription and hormone concentrations, gene products with other physiological functions need at least 10 minutes to become effective for physiological changes (Scherer *et al.*, 2012). *Aux/IAA* genes themselves are auxin-inducible as well. Thus, there may occur a negative feedback loop that ensures a transient response, with the nascent Aux/IAA proteins attenuating the signalling pathway as auxin levels fall by restoring repression of the ARF transciption factors (reviewed in Li *et al.*, 2009; Niklas and Kutschera, 2012). Many genes with auxin-induced expression, such as *SAUR*, *GH3* and *Aux/IAA* genes, share a common sequence in their upstream regulatory regions, TGTCTC (Woodward and Bartel, 2005; Li *et al.*, 2009). As it is discussed below, this *Auxin-Responsive Element* (AuxRE) has been adopted as ‘mould’ to realize the highly active synthetic promoter *DR5* (Ulmasov *et al.*, 1997).

Analysis of endogenous auxin

Auxin is a signal molecule that is present in plant tissues in nanomolar concentrations (Hošek *et al.*, 2012). Therefore, to allow auxin quantification in a restricted amount of material, *i.e.* cell organelles, seedlings, seeds, buds, or apical root- and stem-regions, a sensitive analytical technique is a prerequisite (Prinsen *et al.*, 2000). In the late 30s, the first trials to detect auxin activity were bioassays (Blakesley, 1994). However, the bioassay is not dependent on the IAA amount, but also on the tissue sensitivities. In the early 70s, methods based on color reaction with indole coupled to thin-layer chromatography (TLC)

were developed. This approach permitted the detection of IAA and its metabolites at the same time but nonetheless, the analyses based on the colour reaction were essentially qualitative. During the last two decades, the auxin analysis procedures have been improved substantially. In the late 80s, immunoassay for IAA quantification was developed. However, immunoassay is inconvenient, because IAA must be converted to methyl ester before quantification and in many cases, error range is too wide to trace an endogenous level of IAA. Currently, the most powerful methods to identify IAA and determine the quantity in a single preparation are based on gas chromatography coupled with mass spectrometry (GC-MS), which allows high resolution and sensitivities. Recently, LC-MS (liquid-chromatography coupled to MS) is also applied for the quantification of IAA. Alternatively, cheaper and equally valid IAA quantification methods consist in HPLC (high performance liquid chromatography)-based methods. The use of the reverse phase C_{18} column and well-timed monitoring system do the rest (Kim *et al.*, 2006; Prinsen *et al.*, 2000). However, the use of conventional hormone quantification, *e.g.* enzyme-linked immunosorbent assay or mass spectrometry, does not allow proper study of the internal dynamics of endogenous IAA because spatial information is lost during the extraction step (Thomas *et al.*, 2002; Dong *et al.*, 2011). Blakesley (1994) indicated, among emergent experimental approaches, that immunocytological techniques could be suited to study auxin spatial distribution. In general, the immunolocalisation procedure allows the localisation of plant hormones in tissues and cells using antiplant hormone antibodies, which are subsequently detected using fluorescence-labelled secondary antibodies, and visualised by confocal scanning microscopy. It has been demonstrated that this technique provides results with high sensitivity and resolution and it permits processing either section of embedded material or whole organism (Friml *et al.*, 2003). Recently, visualization of endogenous-auxin distribution by immunolocalisation with IAA antibodies was used in *Arabidopsis* (Aloni *et al.*, 2003), in sun flower (Thomas *et al.*, 2002) and in walnut (Dong *et al.*, 2011). Dong *et al.* (2011) reported that changes in IAA distribution were detected during adventitious root formation in cotyledon explants of walnut. They observed that IAA distribution changed from throughout in freshly excised cotyledons to gradually concentrated in the provascular bundles of the cotyledons, as the differentiation of provascular bundles progressed. When the provascular bundles were fully differentiated, IAA signal in the vascular bundles decreased and became localised

mainly on the periphery of the cells. Afterwards, when the root primordia initiation took place, IAA accumulated strongly in the anular meristematic zones, indicating that IAA is responsible for the formation of root primordia within the anular meristematic zones. Subsequently, in adventitious roots, IAA signal was mainly distributed in the root meristem and root cap. Thus, Dong *et al.* (2011) provided supporting evidence that correlates accumulation of IAA and the induction of either cellular proliferation or determination of developmental fate during advenitious root formation. While immunolocalisation with specific monoclonal antibodies indicated total auxin distribution (both the conjugated and free auxin), another emerging technique allows visualisation of patterns of free-auxin only. Transgenic plants with the bacterial β-Glucuronidase reporter gene (*GUS*) under the control of hormone-sensitive promoters are of special importance. Among them, *DR5::GUS* is the auxin-sensitive one (Ulmasov *et al.*, 1997). *DR5* consists of tandem direct repeats of 11 bp that includes the auxin-responsive TGTCTC element (AuxRE). When *DR5* is fused to the *GUS* gene (hereafter referred to as *DR5::GUS*), the free-auxin distribution can be monitored by analysing the expression of GUS protein, that, during histochemical GUS-staining, readily cleaves X-Gluc (5-bromo-4-chloro-3-indolyl β-D-Glucuronide) to produce intense blue precipitate of chloro-bromoindigo. Hence, sites of free auxin in plants were depicted in blue. *DR5::GUS*-transgenic *Arabidopsis thaliana* plants were used to investigate the distribution of auxin in several studies (Sabatini *et al.*, 1999; Mattson *et al.*, 2003; Aloni *et al.*, 2003; Nakamura *et al.*, 2003; Bai and DeMason, 2008). Bai and DeMason (2008) tested DR5::GUS responsiveness to a range of auxins and to other hormones (such as cytokinins and gibberellins) by exogenous treatments. They found that DR5::GUS *Arabidopsis* plants are responsive to auxins in the order NAA > IAA > IBA. This study proposed that DR5::GUS *Arabidopsis* plants can also be used to verify sites of free auxin following the interaction of auxins and other compounds.

Cytokinins

Cytokinins were discovered by Skoog and co-workers in 1955 (Miller *et al.*, 1955). The hormone received its name because of the ability to activate *in vitro* division (cytokinesis) of plant cells. Since then, cytokinins have been shown to influence plant growth and

development mainly by *de novo* bud formation, release of later buds from apical dominance, promotion of leaf expansion, prevention of leaf senescence, promotion of chlorophyll synthesis, enhancement of chloroplast development in darkness and promotion of seed germination (Gaspar *et al.*, 1996; Mok *et al.*, 2000). In terms of structure, cytokinins include a variety of chemicals, some of which occur naturally in plants and others that are known only as synthetic compounds (Spíchal *et al.*, 2007). Naturally occurring cytokinins are adenine derivatives carrying an isoprene-derived or an aromatic side chain at the N^6 terminus. The isoprenoid cytokinins include N^6-(Δ^2-isopentenyl)adenine (2iP; **fig. 2a**) and cytokinins with a hydroxylated side-chain, such as *trans*-zeatin (**Fig. 2b**), *cis*-zeatin and dihydrozeatin (Mok *et al.*, 2000). Among them, *trans*-zeatin and 2iP are the major forms in *Arabidopsis*, whereas substantial amount of *cis*-zeatin is found in maize, rice and chickpea (Sakakibara, 2006; Hirose *et al.*, 2008). Kinetin (**Fig. 2d**) and N^6-benziladenine (BAP; **fig. 2c**) are the best known cytokinins with aromatic substitutions at the N^6-position, although only BAP and its derivatives were identified as natural cytokinins (Mok and Mok, 2001). Other compounds with cytokinin activity are synthetic derivatives of phenylurea. Diphenylurea (DPU; **fig. 3a**) was the first cytokinin-active phenylurea identified (Shantz and Steward, 1955). This discovery led to the synthesis of a number of potent analogues (Mok and Mok, 2001). Among them, *N*-phenyl-*N'*-(1,2,3-thiadiazol-5-yl)urea (thidiazuron, TDZ; **fig. 3d**) stands out for its cytokinin activity exceeding that of natural compounds (Mok *et al.*, 2000; Sakakibara, 2006, see also below).

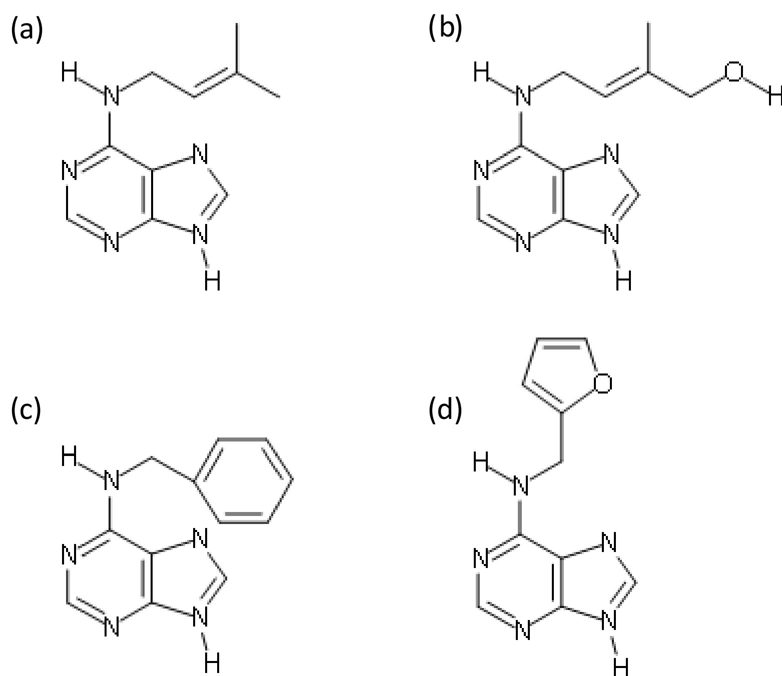

Figure 2
Chemical structure of representative adeninic cytokinins. (a) N^6-(Δ^2-isopentenyl)adenine (2iP). (b) N^6-(*trans*-4-hydroxy-3-methyl-2butenyl)adenine (*trans*-zeatin). (c) 6-benzilaminopurine (BAP). (d) 6-furfurylaminopurine (kinetin).

To regulate such plant developmental processes, cytokinin activity must be finely controlled. Cytokinin activity in an organ is regulated at diverse steps, including *de novo* synthesis, activation, conjugation and degradation.

Cytokinin metabolism

For a long time, cytokinin synthesis has been thought to occur mostly in root tissues. More recently, it has been proposed that it is rather localized in discrete sites throughtout the plant. Nördstrom *et al.* (2004) reported that shoot tissues also have the ability to synthesize cytokinins *de novo*. Cytokinin synthesis is very active in small developing leaves in which a substantial part of the cell still divides. The first step of cytokinin biosynthesis, the transfer of an isopentenyl moiety from dimethylallyldiphosphate (DMAPP) to the N^6 position of ATP/ADP, is catalysed by the enzyme isopentenyltransferase (IPT). The immediate products of the IPT reaction are 2iP ribotides. The isoprene side chain of 2iP ribotides is subsequently *trans*-hydroxylated by the P450 monooxygenases to yield zeatin ribotides. These cytokinin nucleotides can then be converted to their most active free-

base forms via dephosphorylation or deribosylation. Through dephosphorylation, the ribotides are dephosphorylated to the ribosides and subsequently converted to free-base cytokinins. Alternatively, the ribotides can be directly converted to free-base cytokinins by cytokinin nucleoside 5'monophosphate phosphoribohydrolase, LONELY GUY (LOG) (Argueso *et al.*, 2009; Kudo *et al.*, 2010). Degradation is catalyzed by cytokinin oxidase/dehydrogenase (CKO) (Kudo *et al.*, 2010). Because CKOs recognize the double bond of the isoprenoid side chain, aromatic cytokinins are resistant to CKO (Sakakibara, 2006). Cytokinins can be irreversibly inactivated by conjugation to glucose at the N^3, N^7 and N^9 positions of the adenine ring, and can be reversibly modified by conjugation to glucose and, to a lesser extent, xylose, to the hydroxyl group of the side chain (Argueso *et al.*, 2009).

Cytokinin translocation

Cytokinins have been thought to act as local and long-distant signals (Sakakibara, 2006; Hirose *et al.*, 2008). They migrate within a plant along transport channels: in the upward direction from the root into the shoot via xylem and in the downwards and other directions via the phloem. Cytokinin compositions in the xylem differ from those in the phloem (Lomin *et al.*, 2012). In xylem sap, the major form of cytokinin is *trans*-zeatin riboside, and in phloem sap, the major forms are 2iP riboside and ribotide. Thus, it is conceivable that plant might use *trans*-zeatin riboside as an acropetal messanger and 2iP-type cytokinins as systemic or basipetal messengers (Kudo *et al.*, 2010). Cytokinin translocation via the xylem is controlled both by environmental and endogenous signals. For instance, the *trans*-zeatin riboside content and flow rate of the xylem sap are significantly increased by nitrate supplement in barley and maize, implying that *trans*-zeatin riboside acts as a messenger for nitrate signalling (Kudo *et al.*, 2010). Locally, cytokinin transporters are believed to mediate the import and export of cytokinins across the plasma membrane. To date, the purine permease (PUP) family and the equilibrative nucleoside transporter (ENT) family have been proposed to mobilize the cytokinin across the plasma membrane. *Arabidopsis* PUP1 (AtPUP1) and AtPUP2 can transport free-base cytokinins, such as 2iP and *trans*-zeatin, in a proton-coupled manner. Instead, translocation of cytokinin nucleosides, such as 2iP riboside and *trans*-zeatin riboside, is potentially mediated by some ENT family proteins, which facilitate diffusion of

nucleosides along a concentration gradient (Hirose *et al.*, 2008; Kudo *et al.*, 2010).

Cytokinin signalling

Cytokinins are perceived via a phosphorelay that is similar to the two-component regulatory systems with which bacteria sense and respond to environmental stimuli (To and Kieber, 2008). Bacterial two-component system (or histidine-to-aspartate phosphorelay system, His→Asp) consists of two common signal transducers, a sensor exhibiting histidine kinase (HK) activity and a response regulator (RR), which contains a conserved regulatory domain. Once the HK senses extracellular stimuli, it autophosphorylates on a conserved His residue. The RR then catalyzes the transfer of the phosphoryl group from the HK to an Asp residue in its own regulatory domain. Phosphotransfer to the RR results in activation downstream of the effector domain that elicits the specific response (Stock *et al.*, 2000; Suzuki *et al.*, 2001; Takeda *et al.*, 2001). In plants, there is a more complex version of two-component elements that includes two additional phosphotranfers, which occur in sequence His→Asp→His→Asp. Thus, the mechanism consists of HKs, histidine phosphotransfer proteins (HPs) and RRs, resulting in a multistep phosphorelay (Argueso *et al.*, 2009; Kieber and Schaller, 2010).

Cytokinin receptors

The cytokinin receptors were first identified by Kakimoto and co-workers (Inoue *et al.*, 2001). They screened for *Arabidopsis* mutants whose cytokinin responses were impaired. Thus, they isolated a mutant designated *cytokinin response 1* (*cre1*) that displayed insensitivity to cytokinin in root elongation assay. Moreover, *cre1* hypocotyl explants failed to form shoots in conditions (auxin and increasing levels of cytokinin) that induced shoot formation in the wild type. Then, they mapped the *CRE1* locus and it turned out that *CRE1* codes for a histidine kinase. Additional studies published in the same year confirmed the identity of CRE1 (also referred to as AHK4 for *Arabidopsis* Histidine Kinase 4 or WOL for Wooden Leg) as a cytokinin receptor (Suzuki *et al.*, 2001; Ueguchi *et al.*, 2001; Yamada *et al.*, 2001). Subsequent studies identified two additional cytokinin receptors (AHK2 and AHK3) that are present in *Arabidopsis* (Higuchi *et al.*, 2004; Nishimura *et al.*, 2004). AHKs have a complex, multidomain structure and a molecular weight of over 100 kDa. They are hybrid histidine kinases because they contain both the HK domain and the

receiver domain RR. In addition, they possess transmembrane segments and a ligand-binding domain (Shi and Rashotte, 2012). Analysis of primary structures revealed that AHKs are highly homologous to each other (Ueguchi *et al.*, 2001). All three have the ligand binding-domain located at the N-terminus of a receptor molecule, the so-called CHASE domain (Cyclase/Histidine kinase associated sensory extracellular). Sequence comparison revealed that the CHASE domains of CRE1/AHK4 and AHK3 have 65% identical amino acids, while CRE1/AHK4 and AHK2 as well as AHK3 and AHK2, share 63% identity (Spíchal *et al.*, 2004). Studies of the biochemical properties of the AHKs were performed in *Escherichia coli*- or yeast-based test systems expressing individual receptors of *Arabidopsis* (Suzuki *et al.*, 2001; Yamada *et al.*, 2001; Spíchal *et al.*, 2004; Romanov *et al.*, 2006; Stolz *et al.*, 2011). The AHKs complemented yeast and *E. coli* histidine kinase mutants in a cytokinin-dependent manner, providing further evidence of their function (Suzuki *et al.*, 2001; Yamada *et al.*, 2001). Using strains of *E. coli* that express different AHKs, the relative sensitivity of these receptors to various cytokinins at physiological concentrations was also studied (Spíchal *et al.*, 2004; Stolz *et al.*, 2011). Spíchal and co-workers (2004) revealed that 2iP and *trans*-zeatin were highly active in the CRE1/AH4 and AHK3 assays, whereas *cis*-zeatin and dihydrozeatin were recognized only by AHK3. Aromatic cytokinins such as BAP and kinetin had significantly lower activity than isoprenoid cytokinins with both receptors. In addition, *trans*-zeatin riboside had high activity with AHK3 but low activity with AHK4. Of the two phenylurea-type cytokinins tested, DPU and TDZ, only the latter was highly active with both receptors (Spíchal *et al.*, 2004). AHK2 is highly similar to CRE1/AHK4 in ligand preference and function (Stolz *et al.*, 2011). Apart from the difference in cytokinin-binding specificity, cytokinin receptors exhibit a different tissue expression pattern. The CRE1/AHK4 receptor is mainly expressed in roots, whereas AHK3 and AHK2 prevail in leaves and flowers (Lomin *et al.*, 2012; Shi and Rashotte, 2012). In accordance, the effects of cytokinin on the aerial and underground parts of a plant depend, to a larger extent, on the AHK3 and CRE 1/AHK4 receptors, respectively, although the receptors functionally complement each other (Lomin *et al.*, 2012). Recently, the crystal structure of AHK4 CHASE-domain binding to different cytokinins (*i.e.* 2iP, BAP, *trans*-zeatin, kinetin, TDZ) has been determined (Hothorn *et al.*, 2011). AHK4 receptor accommodates chemically distinct adenine-type cytokinins and TDZ in the same site and forms hydrogen bonds with the same amino

acids. Therefore, the basic design principles for active cytokinins require the presence of a planar-ring structure, followed by a linker competent to establish hydrogen bonds and a planar aliphatic or aromatic tail group (Hothorn *et al.*, 2011; Spíchal, 2012). Cytokinin receptors were believed to be localized on the plasma membrane, based on bioinformatic analysis of the protein sequence and analogy with sensor His kinase localization in bacteria and yeast. It was assumed evident that the CHASE domain has to be localized extracellularly, whereas the remaining part of the protein has to be intracellular. The localization of cytokinin receptors on the plasma membrane assumes that cytokinin signal enters the cell from the environment due to extracellular cytokinins. On the other hand, it was demonstrated by determining the pH-dependence of cytokinin binding to receptors that the binding is optimal in neutral and alkalescent media, which are typical of the cytoplasm, and that it decreases abruptly under acidified conditions, which are typical of the extracellular space (the apoplast) (Romanov *et al.*, 2006; Lomin et *al.*, 2012). This suggested that these receptors might function inside the plant cell. Several recent studies have presented evidence supporting the localization of AHKs to the endoplasmic reticulum (ER) membrane (Caesar et al., 2011; Wulfetange *et al.*, 2011; Lomin *et al.*, 2011), although partial plasma membrane localization cannot be ruled out (Wulfetange *et al.*, 2011). In line with these new findings, a modified cytokinin signal phopshorelay model has been proposed. It is proposed that cytokinins enter cell cytoplasm using PUP and ENT channels. Subsequently, cytokinins are directed to the endoplasmic reticulum and activate cytokinin receptors anchored to the membrane (Ciesielska *et al.*, 2012).

Histidine phosphotransmitters

Once HKs autophosphorylate on a conserved His residue in response to cytokinin binding, the phosphate is passed to an Asp in the receiver domain of the HK. Downstream from the receptors, the phosphate is transferred to HPs, which transfer the phosphate to the Asp residues of the RRs localised in the nucleus. Thus, HPs phosphorylate RRs as the target points of the cytokinin signalling pathway (Kieber and Schaller, 2012). HPs are small proteins up to 17 kDa. Similarly to receptors, HP proteins are redundant and participate in the transduction of the cytokinin signal additively. According to current concepts, HP proteins permanently migrate between the nucleus and the cytoplasm. The pattern of their localization is independent of phosphorylation (Lomin *et al.*, 2012).

Response regulators

Based on their protein structure, all 23 *Arabidopsis* RRs (ARRs) were divided into two major groups, type A and type B (Ferreira and Kieber, 2005). Phosphorylated AHPs enter the nucleus and activate the type B ARRs by transferring the phosphate to them. After that, the type B ARRs are released from negative regulator protein and activate transcription of target genes. Such target genes may be type A ARRs or cytokinin response factors (CRFs) (To *et al.*, 2004; Spíchal, 2012). The type B ARRs and CRFs have partially overlapping transcriptional targets that mediate downstream cytokinin-regulated processes including shoot and root development, de-etiolation, leaf-expansion, root vascular differentiation, senescence and cytokinin homeostasis (To and Kieber, 2008). In contrast, type A ARRs act as negative regulators of the initial signal transduction pathway (To *et al.*, 2004; Spíchal, 2012). Phosphorylation of type A ARRs is required for the function in negative feedback regulation, possibly because phosphorylation determines their interaction with other proteins. It has been proposed that type A ARRs activity in feedback regulation may be interfering at the level of AHP-mediated signalling. Hence, the interaction of type A ARRs with AHPs might prevent them from associating with type B ARRs (Choi and Hwang, 2007; Werner and Schmülling, 2009).

Diphenylurea derivatives

In the 1950s, Skoog and co-workers observed that tobacco callus tissue cultures consisting of parenchyma cells but not vascular cells underwent cell enlargement in response to added auxin. However, the cells never progressed to division. Otherwise, when the cells were placed in contact with vascular tissue, some cell divisions occurred in the culture. In the attempt to identify the factors that promote cell division, they supplied coconut milk (a liquid endosperm), yeast extract, or malt extract to the medium. They observed that these substances could mimic the effect of contact with vascular tissue. It turned out that the active materials from yeast extract contained purines. After that, they eventually isolated and named kinetin (**Fig. 2d**; Miller *et al.*, 1955; 1956; Oka, 2003). A parallel effort by Shantz and Steward resulted in the isolation and characterization of one of the cytokinin-active components from coconut milk. They identified the *N*,*N*'-

diphenylurea (DPU; **fig. 3a**; Shantz and Steward, 1955; Oka, 2003). The cytokinin activity of this compound was not high enough to account for the total cytokinin activity present in coconut milk. Moreover, DPU itself has never been detected in coconut milk or any other plant tissue, and it was later found that it was a contaminant from prior chemical analysis (Mok and Mok, 2001). Notwithstanding, this fortuitous finding led to the testing of various synthetic diphenylurea derivatives (Oka, 2003; Ricci and Bertoletti, 2009). The biological activity of about 500 urea derivatives (phenylureas, diphenylureas and thioureas) was then tested (Bruce *at al.*, 1965; Bruce and Zwar, 1966). To determine the biological activity type of newly synthetized compounds, a screening strategy consisting in selective and specific bioassay systems was used. Hence, the authors found some compounds to be consistent with or more active than kinetin. In line with their findings, the authors established the relationships between chemical structure and biological activity (structure-activity relationships) of these compounds:

- DPU is the simplest active compound
- A urea-NHCONH bridge confers higher activity than a thiourea NHCSNH linkage
- Compounds in which both amino hydrogen atoms on either one or both sides of the bridge were substituted are weakly active or completely active
- Among diphenylurea derivatives, compounds with one unsubstituted phenyl ring display the highest activity, and those with two substituted phenyl groups generally have lower activity
- Among phenylurea derivatives, substitution on the ring with phenyl and electronegative groups increases the activity in the order: *meta* > *para* > *ortho* (reviewed in Ricci and Bertoletti, 2009; Yonova, 2010).

In the 1970s, Japanese researchers tested 24 of the highly active urea derivatives reported by Bruce and Zwar (1966) in the tobacco callus assay, and demonstrated that *N*-phenyl-*N*'-(4-pyridyl)urea (4PU; **fig. 3b**) was a hundred times more active than DPU. Therefore, they synthesized 35 N-phenyl-N'-(4-pyridyl)urea derivatives and examined the effects of these compounds in the tobacco callus assay. Thus, they found out that the *N*-phenyl-*N*'-(2-chloro-4-pyridyl)urea (4-CPPU; **fig. 3c**) was 10 times more active than BAP (Takahashi *et al.*, 1978). In the 1980s, Mok and co-workers (1982) reported the discovery of the *Phaseolus* callus growth promoting property of *N*-phenyl-*N*'-(1,2,3-thidiazol-5-yl)urea (thidiazuron, TDZ; **fig. 3d**). In this system, the general trend of activity is *N*-3-

fluorophenyl-*N*'-2-chloro-4-pyridylurea > TDZ > 4-CPPU, with the 4-CPPU activity approximately equivalent to that of zeatin. The authors concluded that there are at least two classes of urea derivatives, the pyridylureas and the thiadiazolylureas, that provide some compounds with high cytokinin activity. More recently, in the attempt to study the cytokinin-like activity of some *N*'-substituted *N*-phenylureas, Ricci and co-workers tested them in various bioassay systems. Among these newly synthesized compounds, the *N*-phenyl-*N*'-(benzothiazol-6-yl)urea (PBU; **fig. 3e**) stood out for its cytokinin-like activity which was even higher than that of TDZ in both the chlorophyll level determination and tomato cotyledon regeneration tests (Ricci *et al.*, 2001a). It has been reported that PBU stimulated also the induction of somatic embryogenesis in stigma and style explants of three *Citrus* species, showing higher embryogenic performance than that of BAP or 4-CPPU (Carra *et al.*, 2006). Very recently, it has been shown that PBU can interact with *Arabidopsis* cytokinin receptor CRE1/AHK4 in a heterologous bioassay system (Rolli *et al.*, 2012).

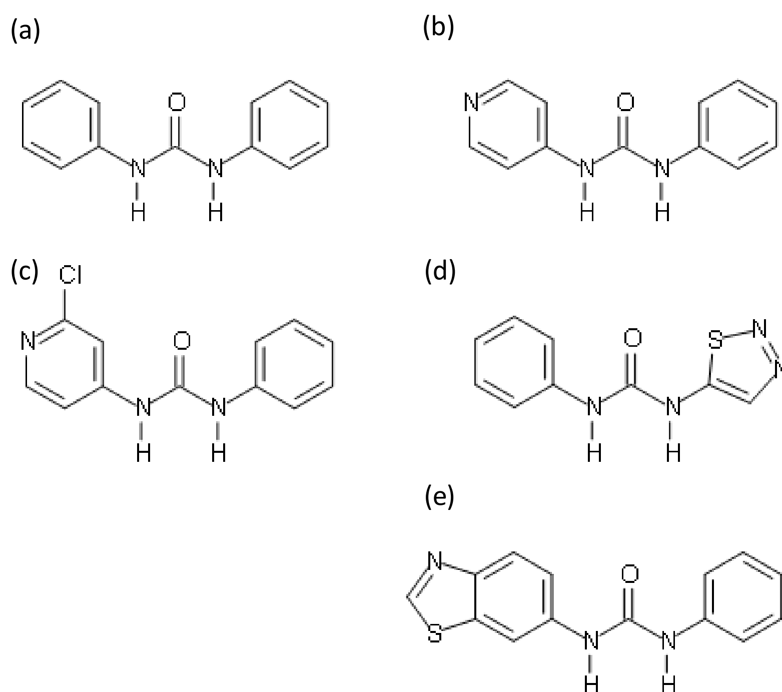

Figure 3
Structures of some synthetic phenylurea cytokinins. (a) *N*,*N*'-diphenylurea (DPU). (b) *N*-phenyl-*N*'-(4-pyridyl)urea (4PU). (c) *N*-phenyl-*N*'-(2-chloro-4-pyridyl)urea (4-CPPU). (d) *N*-phenyl-*N*'-(1,2,3-thiadiazol)urea (TDZ). (e) *N*-phenyl-*N*'-(benzothiazol-6-yl)urea (PBU).

In another study, Ricci *et al*. (2001b) analysed a group of diphenylurea derivatives structurally characterized by:

- Unsubstituted phenyl ring and substituted phenyl ring with a dioxymethylene group linked at 2,3-positions or 3,4-positions
- Unsubstituted phenyl ring and substituted phenyl ring at 2- and 3-position or 3- and 4-position with OCH_3/OCH_3
- The same substitution described above on both phenyl rings (symmetric compounds).

The obtained compounds didn't exert any cytokinin-like activity probably because of the presence of great steric hindrance of the substituents. Surprinsingly, however, *N*,*N*'-bis-(2,3-methylenedioxyphenyl)urea (2,3-MDPU; **fig. 4a**) and *N*,*N*'-bis-(3,4-methylenedioxyphenyl)urea (3,4-MDPU; **fig. 4b**) enhanced adventitious root formation, without showing any cytokinin- or auxin-like activity (Ricci *et al.*, 2001b). Furthermore, similarly to PBU, MDPUs induced somatic embryo formation in *Citrus* species, exceeding the embryogenic activity of BAP and 4-CPPU (Carra *et al.*, 2006). Subsequently, in the strategy of asymmetric and symmetric compounds, the methylenedioxyphenyl group was replaced by isostere benzoxazole or the purine system, and pyrazine derivatives were prepared. It was found out that, among these compounds, 1,3-di(pyrazin-2-yl)urea, 1,3-di(benzo[*d*]oxazol-5yl)urea (5-BDPU; **fig. 4c**) and 1,3-di(benzo[*d*]oxazol-6-yl)urea (6-BDPU; **fig. 4d**) enhanced adventitious rooting without showing cytokinin-like activity, similarly to MDPUs (Ricci *et al.*, 2006). These findings suggested that the enhancement of adventitious rooting activity is rather shown by symmetrically substituted urea derivatives (Ricci *et al.*, 2005; 2006).

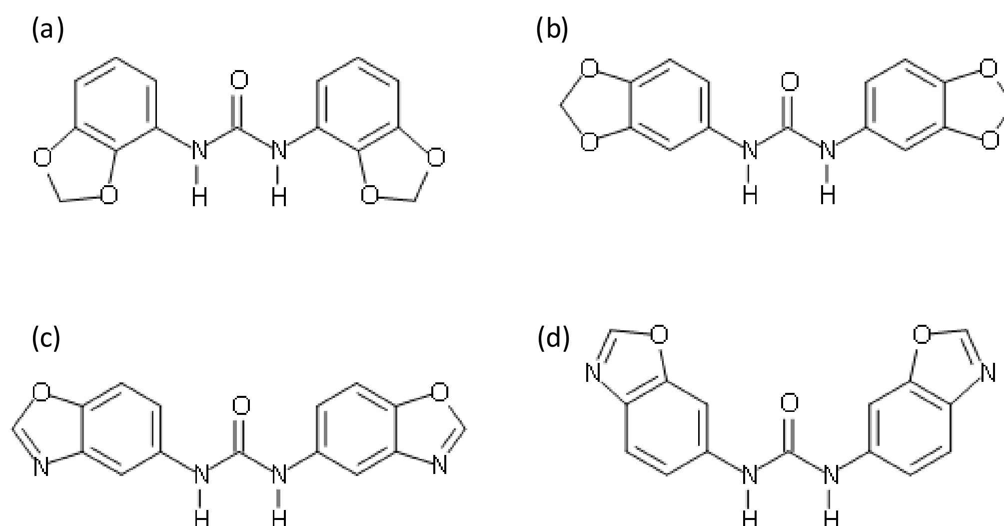

Figure 4
Structures of urea derivatives that act as adventitious rooting adjuvants. (a) *N,N'*-bis-(2,3-methylenedioxyphenyl)urea (2,3-MDPU) and (b) *N,N'*-bis-(3,4-methylenedioxyphenyl)urea (3,4-MDPU), namely MDPUs, have been synthesized from *N,N'*-diphenylurea (DPU) (Ricci *et al.*, 2001b), while (c) 1,3-di(benzo[*d*]oxazol-5-yl)urea (5-BDPU) and (d) 1,3-di(benzo[*d*]oxazol-6-yl)urea (6-BDPU), namely BDPUs, have been synthesized from diheteroarylurea (DHAU) (Ricci *et al.*, 2006).

Adventitious rooting

The evolution of terrestrial plant life was made possible by the establishment of a root system which enabled plants to migrate from aquatic to terrestrial habitats. During evolution, root organization has gradually progressed from very simple to highly hierarchical. Plant anchorage and uptake of water and nutrients from the soil requires an elaborate root system. Types of root systems vary widely either within or between species. According to one of the earliest root biologists, William Canon (1949), root systems can be classified into two groups: those that are derived from the primary root emerging from the seed (primary-root system) and those that arise adventitiously from another organ (adventitious-root system) after germination has taken place. While the primary-root system is characterised by one primary root, from which lateral roots emerge after germination, the adventitious roots, by definition, arise from the shoot, often from the aerial stem and occasionally from the leaves. These roots can perform all the functions of the primary and secondary roots (*i.e.* extraction of water and nutrients from their surroundings) and, in addition, can serve as sites of accumulation of material,

aeration, and traction. In monocotyledonous species, the early root system consists of a seminal root (primary and short lateral roots) and is replaced by crown roots. Because these roots emerge from stem tissue rather than roots, they are by definition adventitious. Therefore, adventitious roots can occur naturally but they may also be induced by mechanical damage or following tissue-culture regeneration of shoots. In the latter two cases, this phenomenon forms the basis of the vegetative propagation via cuttings *in planta* (macropropagation) or micropropagation *in vitro* of many species (Altamura, 1996; Casson and Lindsey, 2003). Vegetative propagation is the bridge between plant improvement and propagation industry of clonally produced plants. Therefore, in horticulture, agriculture, and forestry, it is widely used to multiply elite plants obtained in breeding programs or selected from natural populations (Howard, 1994; de Klerk *et al.*, 1999; Ford *et al.*, 2001). Speed of adventitious rooting is important because rapid rooting minimizes cutting exposure to adverse environments and to the diseases to which unrooted cuttings are prone (Howard, 1994). However, there is dramatic variation between species in shoot cuttings' propensity to form adventitious roots (de Klerk *et al.*, 1999). In general, cuttings from herbaceous species are relatively easy to root, whereas those from woody species are recalcitrant (Hackett, 1988; Altamura, 1996). Hence, poor adventitious root formation and poor quality of the root system is a major obstacle in conventional propagation and in micropropagation. The discovery of the rhizogenic activity of auxin in 1934 is still considered the most significant breakthrough for rooting improvement and plant multiplication (Thimann and Went, 1934; Blakesley, 1994; de Klerk, 2002).

Adventitious root formation

The overall tissue patterning is similar among primary, lateral and adventitious roots (Casson and Lindsey, 2003). Cell types are organised in concentric layers with rhizoderm, cortex, endodermis and pericycle surrounding the central vascular cylinder. In primary root, this radial pattern reflects the arrangement of sets of initials present by the early torpedo stage and is perpetuated through iterative divisions of their respective initial cells, which surround a small group of mitotically less active cells, the quiescent centre (QC), and together with the QC form the stem cell niche at the root tip (Dolan *et al.*, 1993). Roots also exhibited an apical-basal development gradient, with proliferating cells at the

apex and mature cells positioned behind the meristem, in which the newly added cells divide at a lower frequency than in the meristem and grow slowly in length, separated by a zone of expanding cells, where cells undergo rapid elongation, and finally, fully differentiate. (Dolan *et al.*, 1993; Ubeda-Tomás *et al*., 2012). Lateral roots arise typically from the activation of the cell cycle in selected pericycle cells located adjacent to protoxylem poles of the primary root. These undergo a series of periclinal and anticlinal divisions to generate a meristem *de novo* and then the new meristem produces new cells that expand and push the new root tip through the ground and dermal layers to the outside (Casson and Lindsey, 2003; Malamy, 2005). While this organization is quite stereotyped, adventitious roots are less predictable in their cellular sites of origin (Casson and Lindsey, 2003). Most of the time, at the onset of the adventitious root formation in tissue culture, no cells are specified to form adventitious roots. Once the cutting is made, phloem parenchyma or inner cortical parenchyma cells first dedifferentiate. Then, they initiate cell divisions and form adventitious root primordia (Konishi and Sugiyama, 2003). Nevertheless, in some plants, such as willow (*Salix*), preformed adventitious root initials are already present in the stem; however, they are usually very slow to develop and remain dormant; they, then, can develop as adventitious roots only when the stem has been cut and placed in water (reviewed in Blakesley *et al.*, 1991; Geiss *et al.*, 2009). There are at least two pathways by which adventitious roots form: by direct organogenesis from established cell types or from callus tissue following mechanical damage (Altamura, 1996; Casson and Lindsey, 2003). When the rhizogenic process begins with organized divisions in very few competent cells followed by numerous synchronized divisions in localized areas, roots form directly from existing tissue. This is a very regular and precise mechanism (Altamura, 1996; Goldfarb *et al.*, 1998). Since the direct root formation can interest organs with root-like anatomy, such as hypocotyls, this pattern resembles the formation of lateral root meristems in the root tissue of many plants (Goldfarb *et al.*, 1998). In contrast, roots form indirectly through cell divisions of undifferentiated, interfascicular, parenchyma cells following the mechanical damage. In this pattern, dedifferentiation occurs in randomly located cells that are not competent, and cell divisions are not organized, resulting in callus formation (Altamura, 1996, and references therein). Afterwards, callus cells or other parenchyma cells located at the proximal end of the primordium differentiate into vascular elements and provide a connection with

corresponding elements of the initiating organ (Esau, 1977). Dedifferentiation is the early cellular event common to the two patterns and it is necessary in differentiated and differentiating cells for the reactivation of the cell cycle (Altamura, 1996). In herbaceous species, pre-formed root intials are located outside and between the vascular bundles. In cuttings of woody perennials, adventitious roots originate next to, and outward from, the central core of vascular tissues, and, because of the presence of numerous layers of secondary xylem and phloem, usually in the young secondary phloem tissue (reviewed in Geiss *et al.*, 2009). In young hypocotyls of different *Pinus* species, cells that are competent to form roots are precisely positionated in the vascular parenchyma immediately peripheral to the resin canal associated with each of the poles of primary vascular tissue (**Fig. 5**) (Smith and Thorpe, 1975; Diaz-Sala *et al.*, 1996; Goldfarb *et al.*, 1998; Ricci *et al.*, 2008).

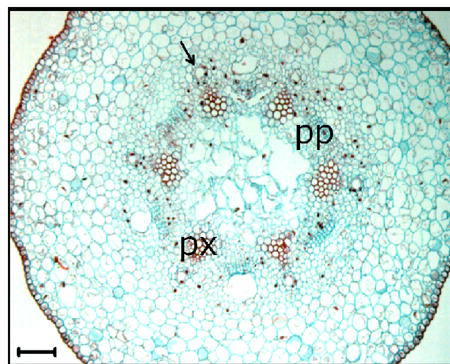

Figure 5
Cross section of hypocotyl cutting from 21-day-old *Pinus radiata* D. Don seedling. The origins of adventitious roots in hypocotyl cuttings are common in different *Pinus* species. The anatomy of the pine hypocotyl is very similar to that of the primary pine root and contains discrete xylem poles, a pericycle, and an endodermis. Root primordia arise from cells in or near the pericycle at the points nearest to the resin canals, which are located exterior to the xylem poles (Goldfarb et al., 1998). pp=primary phloem, px=primary xylem. The arrow indicates resin canal. Bar represents 100 µm. The image was adapted from Ricci *et al*. (2008) with the editor's permission.

Physiological phases and timing of adventitious root initiation

Although adventitious rooting has been considered as a single process for a long time, biological, histological and biochemical evidence accumulated over the years provided arguments leading to a dermarkation of this developmental process into a cascade of steps, each with its hormone requirement (de Klerk *et al.*, 1995; Jásik and de Klerk, 1997; Bellamine *et al.*, 1998; Pop *et al.*, 2011). From a physiological point of view, three phases

have been identified: induction, initation and expression. These three steps can be easily traced back to the main cytological events of the process. The induction phase comprises molecular and biochemical events that lead to nucleus swelling and dense cytoplasm in the cells from which roots will form. Altogether, these events determine the induction of a new meristematic locus. An early transient rise in the concentration of endogenous-free auxin has been described during this period. Such auxin peak is responsible for cell reactivation and it has been casually related to rooting (Blakesley, 1994; Kevers *et al.*, 2009). The initiation phase is defined by cell divisions and root primordia organization. The expression phase is characterized by intra-stem growth of root-primordia and root emergence (Blakesley, 1994; Kevers *et al.*, 2009; Li *et al.*, 2009). During the initiation phase, a very low concentration of endogenous auxin is detected. Such low concentration of endogenous auxin is, then, followed by a gradual increase in the expression phase (Kevers *et al.*, 2009). Other authors referred to these phases in a different way. According to de Klerk *et al.* (1995; 1997), who ascertained the timing of adventitious rooting phases in both apple (*Malus domestica* Borkh. cv. Jork9) microcuttings and stem slices, a previous phase to the induction occurs during the initial 24 hours of the process. They referred to it as 'dedifferentiation', since it is believed that cells become competent to respond to the rhizogenic stimulus of auxin. During this lag period, starch grains begin to accumulate in cells of the cambium, vascular tissues and the primary rays. From 24 to 96 hours, induction took place. By 48 hours, changes in nuclear appearance, increased cytoplasmic density and organelle development coincide with the breakdown of starch grains in these cells. Cambial cells were observed dividing only occasionally at this time. By 96 hours, meristemoids of ca. 30 cells had been formed. From 96 hours (differentiation phase), meristemoids developed into root primordia. After 7 days, the meristems had grown outward from the epidermis of microcuttings, while the primordia emerged from the cutting surface in stem slices (de Klerk *et al.*, 1995; Jásik and de Klerk, 1997). Since no cytological demonstration of dedifferentiation of differentiated cells has been demonstrated to date, the distinction between dedifferentiation and following induction proposed by de Klerk *et al.* is not universally accepted. However, the duration of these phases varies depending on species, genotypes and the culture conditions used**.** In auxin-treated cuttings of *Arbutus unedo*, the induction period was terminated on day 4 from the start of the rooting treatment, whereas, in the case of auxin-treated *Taxus baccata*

cuttings, on day 19 (Metaxas *et al.*, 2004). In hypocotyl seedlings of many pine species, the first cell division in areas of future root meristems occurred between 2-6 days following section of cuttings, rapid cell divisions had not been detected before 6-8 days, while root meristem formation occurred between 10 and 12 days (Smith and Thorpe, 1975; Díaz-Sala *et al.*, 1996; Goldfarb *et al.*, 1998; Greenwood *et al.*, 2001; reviewed in Abarca and Díaz-Sala, 2009b).

Cellular competence and determination

The ability to regenerate roots from somatic differentiated cells gets through to acquisition of cellular competence and determination. These terms describe the state of reactivity and response of cells, tissues or organs to inducing factors (or morphogen). Potential sites for adventitious root initiation in stem cuttings consist of stem cells which switch their identity to give rise to roots (Mohnen, 1994; Wilson, 1994). If a cell has the potential to initiate adventitious root formation, then, this cell is said to be competent for rooting. Competence is acquired before the induction stage (**Fig 6**). Once competent cells have been exposed to the rooting morphogen, they may become determined for root formation (Kevers *et al.*, 2009). Determined cells are, then, committed to a specific developmental fate and they progress into organ formation even after being removed from the root-inducing factor (reviewed in Mohnen, 1994). The irreversible determination state seems to be acquired only after primordium is formed, *i.e.* at the end of the initiation phase or at the beginning of the expression phase, before the emergence of root primordia (**Fig. 6**; Kevers *et al.*, 2009). Experimental manipulation in which tissue explants were firstly cultured in media containing a root-inducing factor, transferred then to a medium without the inducing factor and scored for root formation after a fixed period of time, allowed to define the state of competence and determination during organogenesis (Mohnen, 1994). When competence is achieved during the culture, the pattern of adventitious root formation is indirect. When competent cells are already present in the tissue, the pattern is direct. The time required for determination is dependent on the inducer (*e.g.* type and concentration of auxin used and duration of the culture period), on cellular composition, age of the explant, and on genotype (Altamura, 1996). As suggested by Wilson (1994), competent and non-competent cells exhibit significant differences. Notwithstanding, the basis for cellular competence is still not

known. Because rooting competent and non-competent cells respond similarly during the earliest stages of root induction, it seems that the capacity to re-enter cell division alone is not sufficient to reset the previous cellular state in non-competent cells (Abarca and Díaz-Sala, 2009a).

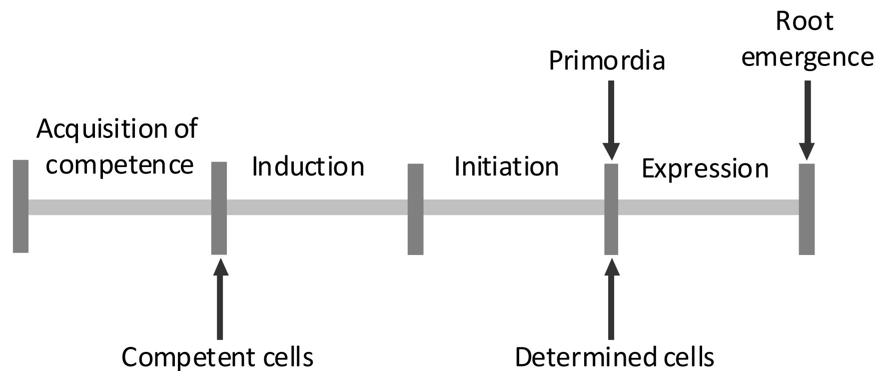

Figure 6
Competence is acquired by few cells prior to inductive phase, whereas the attainment of determination occurs at the end of the initiative phase or at the beginning of the expression. The picture was adapted from Kevers *et al.* (2009) with the editor's permission.

The key role of auxin in adventitious root formation

Auxins profoundly influence root morphology, inhibiting root elongation, increasing lateral root production, and inducing adventitious roots (reviewed in Woodward and Bartel, 2005). For many years, the only evidence of the involvement of auxins in the initiation of adventitious roots came from studies on exogenous applications (reviewed in Blakesley *et al.*, 1991). Depending on the species, age of the plant, season and the culture conditions, application of IBA to cuttings results in the induction of adventious roots, in many cases more efficiently than IAA (Ludwig-Müller *et al.*, 2005; Li *et al.*, 2009). Several possibilities exist to explain the better performance of IBA versus IAA. Biochemical and genetic studies indicate that IBA acts primarly *via* its conversion to IAA. This implies that IBA is a natural occurring slow-release source of IAA (Bartel *et al.*, 2001; Woodward and Bartel, 2005). However, it has also been reported that IBA itself is active (Van der Krieken *et al.*, 1993) and is more stable than IAA both in solution and in plant tissue (Nordström *et al.*, 1991; Ludwig-Müller *et al.*, 2005). Furthermore, differences in their ability to form adventitious roots might rely on differences in auxin metabolism. In sharp contrast with

IAA, IBA is not readily oxidized and is taken up, conjugated, transported more slowly, leaving perhaps more hormone at the plant base where it can affect root initiation (Bartel *et al.*, 2001; Ludwig-Müller *et al.*, 2005). IBA and NAA are employed commercially to induce rooting of cuttings. Auxin may be applied for several days or weeks at a low concentration (micromolar range), or for several seconds or minutes at a high concentration (millimolar range). In both cases, exogenous auxin enters cuttings predominantly via the cut surface and dramatically increases the auxin internal levels. The brief exposure is practiced in macropropagation, in which cuttings are rooted by dipping in a concentrated auxin solution or in talc-based rooting powder. Most likely, auxin is released rapidly from the talc powder (reviewed in de Klerk *et al.*, 1999). In micropropagation, cuttings produced in tissue culture may be treated briefly with auxin, after which they are planted *ex vitro*. When microcuttings are still rather small, it is preferable to root them *in vitro* at a low concentration of auxin and to transfer them to soil after the roots have been formed: during rooting *in vitro*, the microcuttings increase in size and become more robust (reviewed in de Klerk *et al.*, 1999). Exogenous treatments with a high concentration of auxin or treatment with auxin for a long duration may provoke diversion of root primordia formation to excessive xylem differentiation or even prevent rooting, inhibiting outgrowth of root primordial. In fact, external application of auxin stimulates formation of root meristemoids only during the initial steps of rooting (de Klerk *et al.*, 1999; Kevers *et al.*, 2009).

The role of cytokinins in adventitious root formation

Cytokinins synthesized in the root apex and transported through the xylem by transpiration stream are endogenous inhibitors of root formation in the intact shoot. Thus, removal of the root system, which interrupts the flow of cytokinins to the shoot, would allow adventitious root formation. Until the cytokinin content has dropped below a certain threshold level, auxin-stimulated root initiation will not take place (Nordström *et al.*, 1991). The inhibitory effect of cytokinins on rooting has been well documented. *Arabidopsis* mutants with reduced cytokinin synthesis or reduced cytokinin perception displayed increased adventitious root production relative to wild type (Rasmussen *et al.*, 2012). Overexpression of cytokinin oxidase/dehydrogenase that catalyzes the irreversible degradation of cytokinins reduces the endougenous contents of cytokinins, resulting in

enhancement of both lateral and adventitious root formation (Werner *et al.*, 2003). The *ahk4* mutant of *Arabidopsis* is less sensitive to cytokinins than wild type because it possesses a missense mutation in the *AH4/WOL/CRE1* gene which encodes a cytokinin receptor. The phenotype of *ahk4* mutant is characterised by retarded growth of the primary root, absence of lateral roots from the primary root, but elevated number of adventitious roots in the hypocotyl (Inoue *et al*., 2001; Ueguchi *et al.*, 2001; Kuroha *et al.*, 2006). Kuroha and coworkers (2006) observed that *ahk4* mutant has an aborted vascular tissue which consisted of protoxylem only in both hypocotyl (excepted in the upper part of hypocotyl and in the adventitious roots) and primary root. Second, they observed accumulation of auxin in the aborted hypocotyl vascular tissue similar to that observed in the wild type hypocotyl vascular tissue following hypocotyl incision. In the wild type, incision of the hypocotyl led to adventitious root formation. These results suggest that in *ahk4* mutant, the decreased cell numbers in the xylem parenchyma of the hypocotyl determine a reduction of the auxin polar transport (as auxin carriers are localised in the vascular cells) leading to the accumulation of auxin and the activation of adventitious root formation from the hypocotyl. Besides, the lack of lateral root initiation observed in *ahk4* mutant did not depend on the reduction of the vascular-cells number, but it was a consequence of the inhibition of the acropetal auxin transport from the hypocotyl to root, as exogenous application of auxin rescued the lateral root formation. Finally, the evidence that *ahk4* mutant developed a normal vascular system in the adventitious roots suggested that *AHK4* gene is probably not involved in the formation of adventitious roots. However, in the *ahk4* mutant, the alteration of cytokinin responsiveness can inhibit the negative effect of cytokinin in adventititous root formation (Kuroha *et al.*, 2006). The cytokinin-rooting inhibition capacity has been also demonstrated by exogenous application of several cytokinins to apple stem slices (de Klerk *et al.*, 2001). The capacity of applied cytokinin to inhibit rooting formation depends on the 'strength' of the cytokinin; TDZ and BAP showed the highest inhibition, followed by (in order from more effective in rooting inhibition) *trans*-zeatin, kinetin, isopentenyladenosine (IPA), and 2iP. In addition, the same authors reported that 2iP and IPA applied at low concentration in combination with 3 µM IBA, promoted rooting, although only 2iP in a significant way. The time action of 2iP and IPA was specifically during the late dedifferentiation phase or early induction phase (the 2nd day from the excision). Since both 2iP and IPA are considered 'weak' cytokinins, it

has been proposed that low concentration of weak cytokinins or very low concentration of all cytokinins can promote rooting and that all cytokinins at higher concentration become inhibitory to the rooting process. Besides, as high amount of endogenous cytokinin has been observed after the slice excision and this high level is required to achieve rooting, it has been suggested that the presence of 2iP in the medium reinforces the normal requirement of cytokinin necessary to induce the initial cell divisions.

Della Rovere *et al.* (2013) proposed a model of auxin flow, gene expression and cytokinin localisation during adventitious root formation in *Arabidopsis* hypocotyl. Accordingly to this model, at the beginning of the process, IAA is diverted from the basipetal flow along the vascular parenchyma cells adjacent to the protoxylem of the hypocotyl towards the pericycle cells by the PIN1 auxin efflux carrier, activating the LAX3 auxin influx carrier and auxin accumulation in the founder cells. Subsequently, auxin is maintained in the first-formed inner and outer layers of adventitious root by PIN1 and LAX3. PIN1, then, drives auxin flow towards the tip of the adventitious root primordium thoughout the middle cell files, because cytokinin downregulates *PIN1* in the peripheral layers of the adventitious root primordium. Cytokinin also downregulates *LAX3*, limiting the carrier activity at the base of the adventitious root primordium. The auxin flow driven by PIN1 towards the tip results in an apical auxin maximum, establishing here the position of the quiescent center. Biosynthesis of auxin contributes to auxin maximum positioning in the tip. LAX3 is also active in the hypocotyl endodermis, cortex and epidermis around the adventitious root primordium, possibly favouring protrusion. In the mature adventitious root, the auxin maximum encompasses the quiescent centre, flanking initials and cap cells (columella, in particular). Auxin biosynthesis is also maintained, contributing to the persistence of apical auxin accumulation. Also cytokinin is present in the tip of the adventitious root, contributing to the maintenance of auxin homeostasis by downregulation of *PIN1* in the forming epidermis/cortex of *LAX3* in the entire tip, expect the cap. *PIN1* and *LAX3* are expressed in the vasculature of the adventitious root, and *LAX3* expression stops at the elongation zone border.

Urea derivatives as adventious rooting adjuvants

Twelve years ago, neosynthesized *N*,*N*'-diphenylurea derivatives were used in micropropagation of apple cuttings, in order to verify if these compounds were able to promote the formation of adventitious shoots. None of them were effective. However, surprisingly, two of them, *N*,*N*'-bis-(2,3-methylenedioxyphenyl)urea (2,3-MDPU) and *N*,*N*'-bis-(3,4-methylenedioxyphenyl)urea (3,4-MDPU), namely MDPUs, stood out for their ability to induce adventitious root formation (Ricci *et al.*, 2001b). To induce adventitious root formation in apple microcuttings, the best auxinic rooting treatment consisted in culturing the explants with 1µM IBA in the dark, since IBA is a light-sensitive compound. After 6 days of IBA treatment, the microcuttings were transferred to *hormone-free* medium in the light. In this condition, high percentage of rooted microcuttings was achieved and several roots emerged from each microcutting, but callus formation was always present. When MDPUs were tested in the rooting of apple microcuttings, the results were even better than those achieved with IBA. The best rooting stimulation was obtained in the costant presence of 1µM MDPUs in the light without exogenous auxin. Thus, no darkness treatment and no transfer to *hormone-free* media were needed, resulting in a simplified method for rooting apple. Moreover, in this condition, a high percentage of microcuttings rooted and the roots emerged directly from the base of the explants without callus formation. In addition, all the rooted microcuttings survived when transferred to greenhouse conditions (Ricci *et al.*, 2001b). In order to explore their possible auxin-like activity, MDPUs were then tested in rooting of tomato seedlings. To carry out this test, the tomato seedlings were divided into two groups: in one, the seedlings were deprived of their root; in the other one, the seedlings were deprived of both their cotyledons and root. Derooted tomato seedlings with intact cotyledons formed adventitious roots in *hormone-free* condition, suggesting that the auxin produced by their cotyledons is sufficient to lead the process. On the other hand, seedlings deprived of both of their root system and their cotyledons rooted only following exposure to auxin. If MDPUs had auxin-like activity, they would be able to induce adventitious rooting in both groups of tomato seedlings as observed when auxin was applied. Conversely, only the seedlings deprived of their root system showed enhancement in their rooting response following exposure to 5 µM MDPUs. Indeed, MDPUs were unable to increase the root formation in the seedlings deprived of both their cotyledons and root system, suggesting

that intact cotyledons were needed to execute their rhizogenic ability (Ricci *et al.*, 2001b). In the same study, the authors performed other different bioassays in order to verify their possible auxin-like activity. MDPUs were assayed in pea stem elongation test, bioassay for *Pg5-GUS* gene expression as auxins and rooting of apple stem slices (Ricci *et al.*, 2001b; Van der Krieken *et al.*, 1993). The results showed that MDPUs do not behave as auxins. Since these compounds are structurally very close to ureic-type cytokinins, MDPUs were also assayed for their possible cytokinin-like activity in tomato regeneration test, chlorophyll level determination test and bioassay for *Pg5-GUS* gene expression as cytokinins. The results showed that MDPUs do not behave as cytokinins. Thus, the authors concluded that MDPUs might be able to cooperate with molecules originating from other parts of the plants, since these compounds did not show any auxin- or cytokinin-like activity *per se*. In the following study, Ricci and co-workers verified if these two compounds could interact with auxins to enhance adventitious root formation, using stem slices of micropropagated apple shoots (Ricci *et al.*, 2003). The main advantage of this test system is that no compounds originating from other parts of the plant can interfere with auxin-induced root formation (Van der Krieken *et al.*, 1993). Different rooting combinations of MDPUs and low concentration of IBA were tested. The slices were initially cultured with IBA and then transferred in a medium containg MDPUs or *viceversa*, *i.e.* initial treatment with MDPUs and then IBA. Alternatively, the slices were incubated in the simultaneous presence of IBA and MDPUs. Only the latter combination allowed enhancement of root formation in stem slices (Ricci *et al.*, 2003). In light of these findings, it has been proposed that these compounds interact with endogenous auxin in apple microcuttings and with exogenous auxin in apple stem slices. More recently, in order to better characterize the action spectrum of MDPUs, these compounds have been used on the rooting capacity of a herbaceous species (mung bean shoots) and a distantly related woody species (*Pinus radiata* hypocotyls cuttings) (Ricci *et al.*, 2006; 2008, respectively). MDPUs have been reported to increase the adventitious rooting of mung bean plantlets when tested alone and of hypocotyls from 21-day-old seedlings of *P. radiata* when simultaneously combined with IBA (Ricci *et al.*, 2006; 2008). In parallel, in order to verify if MDPUs could be the starting point of a new category of specific compounds with adventitious rooting adjuvant activity, other compounds with chemical modification of the aromatic moiety of MDPUs were designed (Ricci *et al.*, 2006). Thus,

the methylenedioxyphenyl group of the lead compounds (MDPUs) was replaced by the isostere benzoxazole, resulting in the synthesis of 1,3-di(benzo[*d*]oxazol-5-yl)urea (5-BDPU) and 1,3-di(benzo[*d*]oxazol-6-yl)urea (6-BDPU), namely BDPUs. The two new urea derivatives were assayed in adventitious rooting of mung bean shoots and apple stem slices and assessed for their possible cytokinin-like activity using betacyanin accumulation test and tomato regeneration test. Similarly to the lead compounds, BDPUs exhibited a conserved adventitious rooting enhancement, without any biological activity *per se*. Therefore, the substitution of one oxygen with a nitrogen atom in the heteroaryl group preserves the rooting activity enhancement, confirming that the symmetrical presence of heterocyclic rings is an important structural requisite for their activity (Ricci *et al.*, 2006).

Other compounds affecting adventitious rooting

Even if auxin is the real root inducer, other plant growth regulators could display their effects on adventitious rooting together with auxin. Hence, root formation is regulated by a balance between the stimulatory effect of auxin and the effect of other endogenous factors (Ricci and Rolli, 2011).

Ethylene has been implicated in adventitious root formation but its role is still unclear (de Klerk and Hanecakova, 2008; Li *et al.*, 2009). It has been reported that ethylene promotes, inhibits, or does not influence rooting (de Klerk *et al.*, 1999; de Klerk and Hanecakova, 2008; Negi *et al.*, 2010). These contradictory findings may be due to variation in the different tissues, growth conditions, and methods quantifying adventitious root formation (Negi *et al.*, 2010). Ethylene-auxin crosstalk can occur at many different levels since ethylene alters auxin signalling, biosynthesis or transport and in turn auxin can also stimulate the ethylene production (reviewed in Muday *et al.*, 2012). Furthermore, wounding of plant tissues which is usually required to achieve rooting brings about an increase in ethylene. When this increase in ethylene is localized in the lower portion of the stem cuttings, ethylene does not efficiently diffuse out of tissue submerged in acqueous solution or in agar as it is a gaseous compound. As a consequence, it may be accumulated in the rooting zone (de Klerk *et al.*, 1999). Additionally, ethylene induces acidic peroxidases involved in lignin biosynthesis and cellulases, and pectinases that facilitate root emergence through stem tissues. It may also promote rooting by stimulating cytokinin catabolism (reviewed in Li *et al.*, 2009). Ethylene has opposite

effects in the successive phase of rooting, being promotive during the initial stage and inhibitory after that (de Klerk *et al.*, 1999; de Klerk and Hanecakova, 2008).

Not much is known about the role of gibberellic acid (GA) and interaction between auxin and gibberellins during adventitious rooting. Usually, gibberellic acid inhibited, while an inhibitor of gibberellin biosynthesis promoted rooting. It has been reported that application of gibberellic acid to developing adventitious root primordia on stems of *Salix fragilis* reduced the cell number per primordium more in established primordia than during primordia initiation. Thus, it is presumable that gibberellins reduced intraprimordium cell division required for the development of established primordia subsequent to the initiation phase (reviewed in Ludwig-Müller, 2009). Notwithstanding, a pre-treatment of shoots with GA3 sometimes stimulates adventitious rooting, probably because GA3 is involved in the 'rejuvenation process' (reviewed in Ricci and Rolli, 2011). Recently, it has been argued that during the first 3 days after auxin treatment and root induction, transcripts levels of three GA-up regulated genes were increased in hypocotyl cutting from *Pinus contorta* seedlings, suggesting that GA signalling coincided with the activity of the auxin-stimulated cell division (Brinker *et al.*, 2004).

Abscisic acid (ABA) is most likely produced by the cleavage of the xanthophyll. In tissue cultures, treatment with low concentrations of ABA can promote callus growth and organogenesis (buds, roots, embryos), whereas at higher concentrations, it shows inhibitory effect on these processes (Gaspar *et al.*, 1996). Recently, it has been proposed a complex interaction between ethylene, GA and ABA in the control of adventititous root formation in deep-water rice. GA was ineffective on its own, but had a synergistic effect together with ethylene to promote adventitious roots and ABA acted as a competitive inhibitor of gibberellic acid activity (Geiss *et al.*, 2009 and references therein).

Recently, it has been reported that strigolactones, a novel class of plant hormones that were originally discovered for their promotion of mycorrhizal association and parasitic weed seed germination, inhibit the initiation of adventitious roots (Rasmussen *et al.*, 2012). It has been hypothesized that either strigolactone signalling may affect the sensitivity of tissues to auxin or strigolactones may reduce the amount of locally available free auxin, which can then induce adventitious rooting (reviewed in Rasmussen *et al.*, 2013). Strigolactones exert apparently opposing actions in cambial regulation. On the one hand, they reduce the cambial cell division activity required for adventitious root

initiation in stem cuttings. On the other hand, they stimulate secondary growth by promoting cambial cell divisions independent or downstream from strigolatone-controlled auxin accumulation. In light of this, the role of strigolactones may depend on the physiological status of the plant. In addition, it has been proposed that strigolatones may regulate adventitious root development differently in monocots and dicots since impairment of the strigolactones synthesis and perception caused a reduction in rice crown root length. These findings also suggested that not all adventitious roots are in fact physiologically indentical and crown roots may differ from the adventitious roots appearing on non-root tissues in dicotyledonous plants (reviewed in Rasmussen *et al.*, 2013).

Wounding reaction of plant tissue is coupled with synthesis and/or release of many breakdown products. They often did not induce rooting alone but enhanced rooting when applied together with auxin. Therefore, they are thought to play an important role in rooting. de Klerk and colleagues (1999) reported that among wounding-related compounds, polyamines, jasmonic acid, brassinosteroids and phenolic compounds were identified. Their ability to induce adventitious rooting has been studied. Polyamines are involved in cell division and differentiation, thus, in general, they positively correlate to adventitious root formation (reviewed in Ricci and Rolli, 2011). They can also promote or inhibit the adventitious rooting depending on the developmental phases or the species, in both auxin-dependent and -independent manner (Geiss *et al.*, 2009). In fact, recently, it has been reported that exogenous application of putrescine, a diamine, to apple rootstock ‘MM106’ promoted rooting in the absence of auxin, while inhibitors of putrescine inhibited rooting in the presence of auxin (Naija *et al.*, 2009). The role of jasmonic acid and its conjugates on adventitious rooting process is controversial. It has been reported that submicromolar concentrations of jasmonates, combined with root-inductive hormones, promote rooting in apple stem slices and tobacco thin cell-layer explants (TLCs) (de Klerk *et al.*, 1999; Fattorini *et al.*, 2009). However, more recently, Gutierrez *et al.* (2012) proposed that auxin stimulates adventitious rooting by increasing jasmonic acid conjugation and, as a consequence, reducing the free jasmonic acid level in *Arabidopsis* hypocotyls. Therefore, these authors suggested that jasmonic acid homeostasis is under auxin control. In fact, jasmonic acid is a stress-related hormone that can be conjugated by GH3 proteins. Thus, some GH3 proteins may have a role in

controlling the level of free jasmonic acid, at least in hypocotyl tissues. Phenolic compounds, *i.e.* pyrogallol, catechol, phloroglucinol and ferulic acid, are reported to act synergistically with auxin on adventitious rooting (Li *et al.*, 2009). Ferulic acid strongly enhanced rooting of apple stem slices in the presence of IAA and only slightly in the presence of NAA. It has been proposed that it may act as a strong antioxidant, protecting IAA from oxidation (de Klerk *et al.*, 1999). Depending on the time of application, some antioxidants negatively or positively influence rooting. In fact, they inhibited rooting if supplemented during the first phase of the rooting process, whereas they promoted it if supplemented during the successive phases (reviewed in Ricci and Rolli, 2011).

Other factors influencing adventitious rooting

Many other environmental and endogenous factors, such as mineral nutrition, light conditions, temperature, ectomycorrhizas, *Agrobacterium rhizogenes*, plant age and enzymatic activity of peroxidases may affect the adventitious rooting process.

Mineral nutrients are essential in plant metabolism since they can function as constituents of organic structures, as activators of enzymatic reactions, or as charge carriers and osmoregulators. Therefore, it is not surprising that adventitious rooting and mineral nutrition are also intimately related. Nonetheless, only few studies have attempted to characterize the effects of specific minerals on the phases of the adventitious rooting process (Geiss *et al.*, 2009). Schwambach *et al.* (2005) studied the effect of some mineral nutrients on the adventitious rooting response of *Eucalyptus globulus*. They found that root number and root length were significantly affected by mineral nutrition, whereas mean root-time and rooting percentage was affected by auxin availability. Moreover, mineral nutrition influences the root-system quality since the cuttings rooted in an optimized mineral nutrient medium showed higher survival to *ex vitro* acclimatization than cuttings rooted in a basal medium (Schwambach *et al.*, 2005). Calcium ion (Ca^{2+}) is one of the few minerals that can markedly modulate adventitious rooting (Bellamine *et al.*, 1998; Schwambach *et al.*, 2005; Geiss *et al.*, 2009). Ca^{2+} is a second messenger in numerous signalling pathways and changes in its concentration have been reported in response to various signals, including hormones and light (Li *et al.*, 2009). Ca^{2+} is involved fundamentally in cell division and in the root primordia elongation process, which occur during the late rooting phases (Bellamine *et al.*, 1998). It has been

reported that the application of calcium chelators and calcium-channel blocker to poplar shoots negatively affected adventitious rooting *in vitro* (Bellamine *et al.*, 1998). Furthermore, it has been shown that a positive interaction between hormones and specific concentrations of Ca^{2+} improves adventitious rooting (Falasca *et al.*, 2004). However, it seems that there is no general rule, and a positive or a negative effect of one particular nutrient depends on the species and the growth environmental conditions (Geiss *et al.*, 2009).

It is well known that both regulation of auxin metabolism and cell sensitivity to this phytohormone can be affected by light conditions (Fett-Neto *et al.*, 2001). Thus, light conditions might affect adventitious root formation. However, there are a few studies concerning the effect of light on this process. Fett-Neto *et al.* (2001) studied the rooting response of microcuttings of easy-to-root *Eucalyptus saligna* and difficult-to-root *E. globulus* in relation to auxin and illumination. They reported that auxin and light act antagonistically on the development of adventitious roots in both *Eucalyptus* species. The inihibitory effect of irradiance can be explained considering that light can cause photochemical degradation of the medium resulting in decreased availability of auxin and nutrients. It has also been suggested that light may favour cytokinin accumulation in *Eucalyptus*, resulting in suppression of rooting (Fett-Neto *et al.*, 2001). Iacona and Muleo (2010) explored the effect of the light quality on *in vitro* rooting of cherry rootstock. They demonstrated that adventitious rooting was dependent on the light quality to which the microcuttings were exposed. Indeed, exposure to dichromatic light (blue plus red) induced the highest number of roots per microcutting. Moreover, they reported that red light was more effective on root elongation than blue light.

Temperature can potentially influence adventitious rooting capacity in many aspects, such as water and nutrient uptake and metabolism in general, promoting or inhibiting enzymatic action (Geiss *et al.*, 2009). Konishi and Sugiyama (2003) identified temperature-sensitive mutants of *Arabidopsis* defective in various stages of adventitious rooting formation, confirming that temperature can essentially influence the different phases of the process. Nevertheless, the role of temperature in controlling the rooting process is not clear yet and has still to be elucidated (Geiss *et al.*, 2009).

Ectomycorrhizas, *i.e.* symbiosis formed between certain fungal and plant species, can stimulate development and growth of roots. Therefore, inoculation with specific

ectomycorrhizal fungi has been tested to stimulate adventitious root formation of cuttings of difficult-to-root species. It has been reported that the fungal-induced rooting responses resemble those caused by exogenous auxins (Niemi *et al.*, 2002). As a matter of fact, several ectomycorrhizal fungi can synthesize free IAA and the stimulation of root production has been associated with the production of free IAA by the fungus (Niemi *et al.*, 2002). Furthermore, some ectomycorrhizal fungi are able to produce and release other rooting compounds, such as putrescine and cadaverine (reviewed in Geiss *et al.*, 2009).

Agrobacterium rhizogenes is a plant pathogen whose infection is characterized by a massive production of adventitious roots. Root induction is due to the integration and expression of the T-DNA from the root-inducing Ri plasmid in the plant genome. The T-DNA harbours four root loci (*rol*) genes: *rolA*, *rolB*, *rolC* and *rolD*. The *rolB* gene was identified as the critical *rol* gene for the induction of roots, because it can individually induce adventitious root formation in different plant species. Nevertheless, maximum adventitious rooting occurs when the *rolB* gene is combined with either *rolA* and/or *rolC*, suggesting that the three genes synergistically control the rooting response. Therefore, *A. rhizogenes*-mediated transformation has been used successfully to enhance adventitious rooting in several fruit species (reviewed in Geiss *et al.*, 2009).

Adventitious rooting potential of many woody species, particularly tree species, may decrease during ontogenic aging and maturation. In the development of all woody plants from seed there is the so-called 'juvenile phase', during which flowering does not occur because the ability to flower is achieved and mantained only in the so-called 'mature phase'. The transition from juvenile to mature condition is generally associated with gradual changes in morphological, developmental, and physiological characteristics which may be difficult to detect. Many tree species exhibit a high adventitious rooting ability in the juvenile phase, while it is lost with increasing age of seedling derived mother plants. Therefore, the competence to form adventitious roots is considered as a juvenile characteristic (Hackett, 1988). The rate and the extent of the loss of competence to form adventitious roots are species dependent. The dramatic effects of plant maturation are expressed as a lower percentage of cuttings forming roots, fewer roots induced per cutting and increased time for root emergence. In many species, such as *Eucalyptus*, juvenility is retained in the lower portion of the tree (Hackett, 1988). An experimental

rooting system in which both easy-to-root juvenile and difficult-to-root mature tissues cohabit in the same individual can be used to understand the mechanisms controlling root formation in stem cuttings of many conifer species (Goldfarb *et al.*, 1998). Díaz-Sala *et al.* (1996) studied the effect of maturation on rooting capacity of stem cuttings made from hypocotyls and epicotyls from 50-day-old seedlings of *Pinus taeda* L. (loblolly pine). They observed a dramatic decline of rooting ability by epicotyl cuttings, which did not root at all in 20-30 days in the presence or absence of auxin. In contrast, almost all the hypocotyl cuttings rapidly formed adventitious roots in the presence of exogenously applied auxin. The lack of root initation by epicotyls was not related to difference in auxin uptake, transport, metabolism or distribution in the two types of cuttings. At the cellular level, epicotyl cuttings underwent the same initial response to exogenous auxin as hypocotyl ones, because differentiation of the cambium into parenchyma occurred in both types of cuttings. Exogenous auxin uptake in cuttings is through the transpiration stream, entering mostly through the base of the cutting, and is loaded on basipetal transport. Thus, polar auxin transport is essential for the process. The role of polar auxin-transport on the rooting process, therefore, has been studied. The requirement of polar auxin transport occurred only during the initial 48 hours of the induction process. Afterwards, polar auxin transport did not have any effect on the rooting process (Díaz-Sala *et al.*, 1996; 2002). These findings indicate that auxin does not account for all the developmental variation in rooting ability, and rather this capacity is related to the state of competence of cells, which probably lacks in epicotyls (Greenwood *et al.*, 2001). It is not known whether this lack of response by epicotyls is due to a loss of specific cell type, the inability of individual cells to perceive auxin signals specific for root meristem organization, the suppression of gene expression needed for cells to enter the root formation pathway, or chromatin status and epigenetic mechanisms resulting in a specific nuclear architecture involved in the control of age-dependent cellular plasticity (Hutchinson *et al.*, 1999; reviewed in Abarca and Díaz-Sala, 2009a; 2009b). In the studyby Ricci *et al.* (2008), combinations of auxin and symmetrically substituted urea derivatives, such as MDPUs, have been tried for their hypothetical ability to reverse the retention of rooting ability of both hypocotyl and epicotyl cuttings of 56-day-old *Pinus radiata* seedlings, which showed a low or null competence to root in the presence or absence of auxin (Ricci *et al.*, 2008; Sánchez *et al.*, 2007). MDPUs did not induce a reversal of the

competence loss in both types of non-competent cuttings. However, when combinations of MDPUs and IBA were tested on the rooting capacity of competent hypocotyl cuttings from 21-day-old *P. radiata* seedlings, MDPUs magnified the response to auxin stimulus. This behaviour had positive related effects: the use of low auxin concentrations to stimulate adventitious root formation, the emergence of roots directly from the base of the cutting, the absence of callus at the emergence site, and last but not least, a good quality root system. At the cellular level, these compounds did not affect the competence for rooting of cell types other than those that retained intrinsic competence to form adventitious roots in response to auxin. It has been hypothesized that the enhancement observed when MDPUs were simultaneously supplied with low concentration of exougenous IBA could be due to local changes in auxin distribution (Ricci *et al.*, 2008).

Classical plant peroxidases are heme-containing enzymes that catalyzed the oxidation of diverse organic compounds. Peroxidases exist in numerous isoenzymatic forms and are separated into anionic (acidic) and cationic (basic) types according to their isoelectric points. Peroxidases have a major role in hormone catabolism, such as decarboxylative catabolism of IAA (Li *et al.*, 2009). The auxin transported basipetally in the shoot, consequently when a cutting is excised, accumulated at the base. This accumulation is aided by reduced activity of IAA oxidase/peroxidase in the rootng zone (Blakesley *et al.*, 1991). In addition, changes in peroxidase activity have been proposed as biochemical markers of the successive rooting phases (Metaxas *et al.*, 2004; Li *et al.*, 2009). During the progress of the adventitious rooting phases, the endogenous levels of free IAA and the peroxidase activity have opposite trend. The induction period is characterized by a sharp drop in the peroxidase activity whilst the IAA levels reach a peak. Afterwards, the IAA/peroxidase activity pattern reverses. Thus, the initiation phase is characterised by a reduction of endogenous IAA concentration and a gradual increase of peroxidase activity. When the peroxidase activity peaks, the expression phase starts. Then, as the expression phase progresses, the peroxidase activity gradually declines and IAA accumulates (Kevers *et al.*, 2009).

Genes involved in the control of adventitious root formation in woody species

Technical limitations imposed by long regeneraton time, lack of mutants, problems in transformation and regeneration procedures make difficult to study the molecular

mechanisms underlying the capacity to form adventitious roots in woody species (Díaz-Sala *et al.*, 2002). Therefore, in order to extend our knowledge of the genes related to the adventitious rooting process in woody species alternative techniques should be considered. Using differential display reverse transcription-polymerase chain reaction (RT-PCR), Hutchinson *et al.* (1999) identified a gene family whose mRNA levels increased in the first 24 to 48 hours in hypocotyl cuttings from loblolly pine seedlings in response to the application of auxin. This gene family encodes expansins which are thought to be responsible for acid-induced loosening of cellulose-hemicellulose networks. More recently, the gene expression pattern during adventitious rooting of *Pinus contorta* hypocotyls was examined by microarray analysis (Brinker *et al.*, 2004). A significant change in the transcript level of 220 genes could be observed over the period of root development. During the root initiation phase, genes involved in cell replication, cell wall weakening and a *PINHEAD/ZWILLE-like* gene were upregulated, while genes related to auxin transport, photosynthesis, and cell wall synthesis were downregulated. In contrast, during the root meristem formation phase, genes involved in auxin transport and cell wall synthesis were upregulated, while those related to cell wall modification were downregulated. Finally, during the root elongation phase, genes encoding proteins involved in cell replication were downregulated. In apple, expression of the *Adventitious Rooting Related Oxygenase* (*ARRO-1*) gene is upregulated 24-72 hours after the onset of the rooting process in response to exogenously applied auxin (Butler and Gallagher, 2000). In an attempt to better understand the role of *ARRO-1* in adventitious root formation in apple, Smolka *et al.* (2009) used the RNA interference (RNAi) technique to transform apple rootstock M26. RNAi represents a research tool to induce suppression of specific genes of interest by causing destruction of specific mRNA molecules. The transgenic clones obtained by silencing *ARRO-1* gene through RNAi therefore exhibited downregulation of *ARRO-1* expression. These clones appeared to be more sensitive to exogenous hormones compared to untransformed control plants. Hence, the authors proposed that *ARRO-1* is involved in adventitious root initiation, probably through regulating hormone homeostasis. Sánchez *et al.* (2007) characterized two closely related *SCARECROW-like* (*CsSCL1* and *PrSCL1*) with possible roles in the formation of adventitious roots in two distantly related species, *Castanea sativa* and *Pinus radiata*. In addition, the same authors reported the characterization of the *PrSHR* gene, the putative ortholog to

the *Arabidopsis thaliana SHORT-ROOT* gene, in *P. radiata* (Solé *et al.*, 2008). SCARECROW, SCARECROW-like and SHORT-ROOT proteins are putative transcription factors and they are involved in the radial patterning of roots, hypocotyls and aerial organ (reviewed in Abarca and Díaz-Sala, 2009b; see also below). In an attempt to understand the role of *PrSCL1*, *PrSHR* and *CsSCL1* during the adventitious rooting process, analysis of their expression and localization of their mRNA in rooting-competent and -incompetent cuttings from both pine and chestnut have been carried out (Sánchez *et al.*, 2007; Solé *et al.*, 2008; Vielba *et al.*, 2011). In chestnut, rooting-competent shoots were obtained from basal shoots of the mature tree, and needed auxin exposure to root. Conversely, those collected from the crown of the same tree are rooting-incompetent and despite auxin treatment, they root poorly. Differences in *CsSCL1* expression between basal and crown microshoots have been detected at the end of the multiplication cycle prior to auxin treatment, suggesting that *CsSCL1* expression is dependent on the shoot ontogenetic stage (Vielba *et al.*, 2011). Exogenous application of auxin to chestnut microcuttings induced cell division in either two types of explants but results in adventitious root development in basal shoots only. *CsSCL1* expression was up-regulated by auxin in the basal portion of both basal and crown shoots during the first 24 hours of treatment, when cell dedifferentiation and early mitotic activity occurred (Sánchez *et al.*, 2007; Vielba *et al.*, 2011). The presence of early cell division in both root-competent and -incompetent explants supported the finding that reactivation of cell division alone cannot be considered as the initiation of root meristemoids (Díaz-Sala *et al.*, 1996; Greenwood *et al.*, 2001; Vielba *et al.*, 2011). Interestingly, *in situ* hybridisation analysis revealed different expression patterns of *CsSCL1* in basal and crown microshoots in response to auxin. *CsSCL1* mRNA localised throughout and no-specifically in basal portion of crown shoots after 12, 24, and 48 hours of treatment with IBA, probably related to callus formation, whereas *CsSCL1* transcripts localised specifically in the cambial zone and derivative cells (*i.e.* competent cells) 12 hours after IBA treatment, and accumulated throughout the phloem cells 24 and 48 hours following IBA exposure. Afterwards, *CsSCL1* transcripts were restricted to the root primordia developed in IBA-treated basal shoots by 7-9 days after root induction. As primordia developed, *CsSCL1* transcripts accumulated in the inner cell layers and ground tissue of the adventitious root with low levels of mRNA. Collectively, these results are consistent with a direct relationship between *CsSCL1* mRNA levels

asymmetrically localised to specific cell types in IBA-treated basal microshoots, and root primordia development. Hence, *CsSCL1* may determine whether certain cells will enter the root-differentiation pathway and give rise to meristem initial cells of the adventitious root. The localized expression of *CsSCL1* in the procambial cells of IBA-treated basal microshoots, but not in those of crown microshoots, appears to be critical for the adventitious rooting process. On the other hand, in IBA-treated crown microshoots, the diffuse and non-specific expression of *CsSCL1* may be related to callus formation (Vielba *et al.*, 2011). In Monterey pine, hypocotyl cuttings of 21-day-old seedlings are rooting-competent explants and root only following treatment with IBA, whereas hypocotyl and epicotyl cuttings of 56-day-old seedlings show low or null competence to root. *PrSCL1* expression increased in response to exogenous auxin within the first 24 hours of the root induction process in the cambial region of basal portion of rooting-competent cuttings (Sánchez *et al.*, 2007). Conversely, *PrSHR* transcript accumulated within the first 24 hours after primary-root excision in both auxin-treated and -untreated pine hypocotyl cuttings (Solé *et al.*, 2008). This is consistent with the finding that *MtSHR1* transcript (*Medicago truncatula SHR1*) was not accumulated following treatment with NAA during *in vitro* root formation of *M. truncatula* leaf explants, while *MtSCR1* transcription was positively affected by auxin treatment (Imin *et al.*, 2007). Furthermore, in the 21-day-old seedlings, *PrSHR* expression is predominantly confined to root and with a lesser extent to other organs, such as hypocotyl, shoot apex, and cotyledons (as *PrSCL1* expression profile). In the basal portion of 21-day-old hypocotyl cuttings, *PrSHR* transcripts were abundant in the cambial region 24 hours after excision of the primary root. Its transcript accumulation was not found in older root-incompetent hypocotyls (Solé *et al.*, 2008). In other plant systems, *SHR* is expressed before root meristem initiation (Helariutta *et al.*, 2000). Therefore, it has been suggested that, in contrast to *PrSCL1*, *PrSHR* expression may be an exogenous auxin-indipendent adventitious rooting competence factor (Solé *et al.*, 2008). Taken together, these findings led to speculation that, since there was an overlap in the temporal and spatial expression domain of both genes in competent cuttings, *PrSCL1* and *PrSHR* may have a possible function in the same tissues at the same time (Solé *et al.*, 2008; Abarca and Díaz-Sala, 2009a; see also below). The expression of *PrSCL1* can be also affected when rooting-competent *Pinus radiata* cuttings were exposed to a mixture of auxin and MDPUs (Ricci *et al.*, 2008). Indeed, *PrSCL1* transcript levels significantly

increased during the early stage of adventitious root formation in the presence of a low level of IBA plus 2,3-MDPU compared to expression levels recorded in the presence of IBA alone, suggesting that MDPUs can affect the auxin signalling pathway. Furthermore, this mixture significantly decreased the *PrSHR* transcript abundance with regard to IBA alone within the first 24 hours of the root induction process (Ricci *et al.*, 2008).

Scarecrow and shortroot

As already mentioned, the root consists of a radial organization of cell files in which, in order from the outermost layer, rhizoderm, cortex and endodermis surround the central stele, which consists of the pericycle, phloem and xylem. This radial-tissue pattern is perpetuated through iterative divisions of their respective initial cells, which surround a small group of mitotically less-active cells, the quiescent centre (QC), and, together with the QC, form the stem-cell niche at the root tip. The cortex and endodermis, collectively called the ground tissue, are derived from the cortex/endodermis initial cell through two consecutive asymmetric cell divisions (**Fig. 7**). The cortex/endodermis initial cell first divides anticlinally (in a transverse orientation), giving rise to two daughter cells with different fates: the cell adjacent to the QC remains a stem cell, whereas the other divides periclinally (in a longitudinal orientation) to produce the first cells of the two lineages.

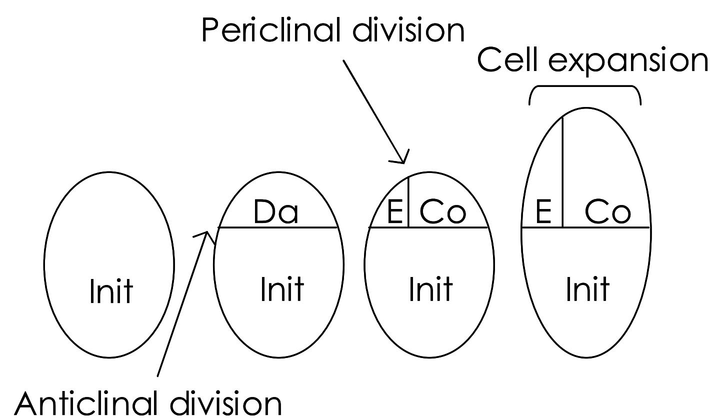

Figure 7
Schematic representation of division pattern of the cortex/endodermal initial. The cortex/endodermis initial expands and then divides anticlinally to reproduce itself and a cortex/endodermis-initial daughter-cell. The daughter then divides periclinally to reproduce the progenitors of the endodermis and cortex cell-lineages. Subsequently, the progenitor cells undergo expansion. Init=initial; Da=daughter; Co=cortex; E=endodermis. The picture was taken from Di Laurenzio *et al.* (1996) with the editor's permission.

SHR and SCR are regulatory proteins (transcription factor), which control the asymmetric cell divisions required to establish the pattern of the ground tissue. In *shr* and *scr* mutants, the root length was greatly reduced compared with wild-type (Benfey *et al.*, 1993; Scheres *et al.*, 1995). In both these mutants, the cortex/endodermal initial divides anticlinally as the wild type, but the subsequent periclinal division that increases the number of cell layers does not take place, resulting in a single layer of ground tissue (Benfey *et al.*, 1993; Scheres *et al.*, 1995). This radial-organization defect in *scr* is already evident during embryogenesis (Scheres *et al.*, 1995; Wysocka-Diller *et al.*, 2000) and is not limited to the primary root, but it is also present in lateral roots (Di Laurenzio *et al.*, 1996; Malamy and Benfey, 1997), in adventitious roots regenerated from calli (Di Laurenzio *et al.*, 1996) and in adventitious roots originated from hypocotyl explants (Konishi and Sugiyama, 2006). Although both *scr* and *shr* mutants have the ground tissue formed by a single cell layer, instead of two, as a consequence of the lack of the periclinal asymmetric cell-division, they differ from each other in the identity of the resulting mutant-cell layer (Di Laurenzio *et al.*, 1996). The *SHR* gene is essential for cell division and is involved in specification of endodermis indentity. Thus, the single layer of the ground tissue in the *shr* mutant possesses attributes of the cortex (Benfey *et al.*, 1993). By contrast, since the mutant-cell layer in *scr* has differentiated characteristics of both cortex and endodermis, *SCR* has a role in the regulation of the asymmetric cell division of the cortex/endodermal initial rather than in specification of the identity of either cortex or endodermis (Di Laurenzio *et al.*, 1996). Moreover, it has been also proposed that SCR, in combination with auxin-dependent cues, control stem cell identity, since *SCR*-expressing cells are competent to acquire QC indentity (Sabatini *et al.*, 2003). The *SCR* gene is expressed in the QC (Wysocka-Diller *et al.*, 2000; Sabatini *et al.*, 2003), in the initial daughter cell before its asymmetric division and remains expressed in the endodermal cell layer after the division (Di Laurenzio *et al.*, 1996; Wysocka-Diller *et al.*, 2000), whereas *SHR* is not expressed in the ground tissue cell lineage but in the stele (Helariutta *et al.*, 2000). Once *SHR* has been expressed in the stele, the SHR protein moves into the adjiacent cell layer where cortex/endodermis initial cells reside, and activates the programs for asymmetric cell division and endodermis specification (Nakajima *et al.*, 2001). The nuclear localisation of SHR in the cortex/endodermis initials promotes activation of *SCR* expression (Nakajima *et al.*, 2001). The SHR upstream regulation of *SCR* has also been observed in *scr* mutation,

which has no effects on *SHR* expression but, instead, in the *shr* background, the expression of SCR was consistently reduced (Helariutta *et al.*, 2000; Cui *et al.*, 2007). Besides, SCR is able to bind to its own promoter, suggesting SCR autoregulation controlled by SHR/SCR-dependent positive feedback loop (Cui *et al.*, 2007). The positive feedback loop for SCR transcription is required to completely block SHR movement and it has been shown that SCR directly binds to SHR rather than to itself (Cui *et al.*, 2007; Lee *et al.*, 2008), resulting in expression of the SCR/SHR target genes (Cui *et al.*, 2007). The functional relationship between SHR and SCR, as well their role in radial patterning in higher plants, is evolutionary conserved (Cui *et al.*, 2007). Recently, it has been shown that, while SCR is required for the asymmetric division of the cortex/endodermis-initial daughter, it represses subsequent divisions, resulting in the characteristic two cell-layer ground tissue. These two opposing functions of SCR are executed by different parts of the protein through interaction with various partners: asymmetric cell division is activated by the C-terminal region interacting with SHR, but is repressed by the N-terminal variable domain through physical interaction with the LIKE HETEROCHROMATIN PROTEIN 1 (LHP1) protein, which may be able in turn to reverse the epigenetic markers in chromatin (Cui and Benfey, 2009). SCR, SCL and SHR belong to the plant-specific GRAS transcription factor family, named after the three founding members, GIBBERELLIC ACID INSENSITIVE (GAI), REPRESSOR OF GAI (RGA), and SCARECROW (SCR). GRAS proteins have been found in many higher plants. Typically, GRAS proteins exhibit considerable sequence homology to each other in their respective C-termini (**Fig. 8**). The distinguishing domains of GRAS proteins are two leucine-rich (L-rich) areas flanking a VHIID motif. The two L-rich domains may be involved in protein-protein interaction (Bolle, 2004). For instance, it has been proposed that the two-leucine heptad repeats and the VHIID motif is responsible for the above-described SHR and SCR interaction (Cui *et al.*, 2007). Several additional amino-acid residues are invariant in most of the GRAS protein family. These include PFYRE and RVER motifs (**Fig. 8**). Despite the substantial homology between GRAS proteins in the C-terminal part, the N-terminal amino-acid sequences are highly divergent (**Fig. 8**). Moreover, several members of the GRAS-protein family contain the nuclear localization signals (NLS), indicating that these proteins may accumulate in the nucleus. Nonetheless, LlSCL (*Lilium longifolium* SCL) has been reported to localize exclusively in the nucleus and has a role in the transcription regulation, though lacking in classical NLS. Besides, GRAS proteins have

parallels in their domain structure to STAT animal proteins, which have been shown to bind DNA. In light of these findings, it has been proposed that GRAS members act as transcription factors (reviewed in Bolle, 2004).

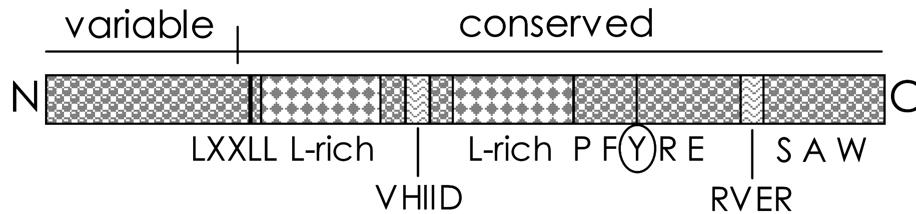

Figure 8
Schematic presentation of the domain structure of GRAS proteins. Conserved domains and amino-acid residues are indicated. The picture was taken from Bolle (2004) with the editor's permission.

The GRAS-protein family can be divided into several clades, which have been designated after one of their members or a common feature: the DELLA proteins, the SCR branch, the Ls branch, the HAM branch, the PAT1 branch, the SHR branch, and the SCL9 branch (Bolle, 2004). Unlike *SCR*, which contains a single intron, the genomic sequences for several SCLs (including *SCL9*) all appear to contain a single open-reading frame encompassing all of the motifs present whithin the GRAS family. Moreover, the majority of *SCL* genes are expressed predominantly in the root (Pysh *et al.*, 1999). The deduced amino-acid sequences of PrSCL1 and CsSCL1 showed complete conservation at the carboxyl-terminal region with the characteristic features of SCR and other members of the GRAS-protein family that point to their potential role as transcriptional activators, indicating conserved functions of these proteins (Sánchez *et al.*, 2007). A phylogenetic analysis indicated that PrSCL1 and CsSCL1 form a single clade with the SCL9 branch, showing the highest similarity to LlSCL (Sánchez *et al.*, 2007). Likewise, the deduced amino-acid sequence of PrSHR contained domains characteristic of SHR proteins and, as suggested from phylogenetic analysis, it formed a single clade with the AtSHR branch, to which it showed the highest similarity (Solé *et al.*, 2008). Thus, the phylogenetic analysis suggested that these genes may control the tissue reorganization similarly to their homologous in *Arabidopsis*.

Somatic embryogenesis

Life strategies of multicellular plant and animal are markedly different. While movement is recognized as the basis of animal behaviour, higher plants are sessile. Therefore, for most plants the only available forms of action are either growth or discarding of parts, both of which involve a change in the size and form of the organism by mostly altering metabolism and/or development (Fehér *et al.*, 2003; Trewavas, 2009). This capacity of a given genotype to express different phenotypes in different environments is known as phenotypic plasticity (Sultan, 2000). Thus, the ontogenic program of plants is highly flexible and this is linked to the reversibility of the differentiation state of somatic plant cells (Fehér *et al.*, 2003). Under extreme conditions, these cells have to switch their fate into a multipotent (*i.e.* can form multiple lineages that constitute an entire tissue or tissues), pluripotent (*i.e.* able to form all the body's cell lineages) or totipotent (*i.e.* sufficient to form entire organism) cells that can develop a root, shoot or embryo or repair damaged tissues (Abarca and Díaz-Sala, 2009a). One of the most extreme examples of flexibility in plant development is the capability of somatic cells, in addition to the zygote, to initiate embryogenic development. The ability to produce morphologically and developmentally normal embryos and, indeed, whole plants from undifferentiated somatic cells in culture, through the process of somatic embryogenesis, resides uniquely within the plant kingdom (Zimmerman, 1993). In seed plants, embryos and seeds can be generated without fertilization through various pathways, which are collectively referred to as apomixis. The term apomixis describes the formation of an embryo in the ovule from somatic cells. Embryogenesis can arise from isolated somatic or gametic (microspore) cells, either naturally, as has been observed in *Kalanchöe*, where somatic embryos form spontaneously on the edge of leaves, or *in vitro* after experimental induction (Dodeman *et al.*, 1997). However, somatic embryogenesis occurs to a limited extent under natural conditions. Nonetheless, it can probably be achieved for all plant species provided that the appropriate explant, culture media and environmental conditions are employed (von Arnold *et al.*, 2002). *In vitro* embryogenesis has therefore practical and commercial applications, particularly for *in vitro* clonal micropropagation. In some cases, somatic embryogenesis is favoured over other methods of vegetative propagation because of the possibility to scale up the propagation by using bioreactors. Other practical applications

include the production of synthetic seed, crop improvement (cell selection, genetic transformation), germplasm preservation (through cryopreservation), virus elimination, *in vitro* metabolite production, and *in vitro* mycorrhizal initiation (Vicient and Martínez, 1998). In addition, *in vitro* embryogenesis can serve as a model system to study the molecular, cytological, physiological and developmental events underlying plant embryogenesis. The main advantage of these *in vitro* experimental systems is that embryogenic cells are accessible for manipulation by most cellular and molecular techniques, in contrast to gametic cells and zygotes, which develop embedded in maternal tissues (Fehér *et al.*, 2003). Since woody plants are generally considered to be recalcitrant to tissue culture, somatic embryogenesis for the production of synthetic seeds is a potential resource for biotechnological applications in clonal forestry. For conifers, somatic embryogenesis currently involves the culture of zygotic embryos, from which virtually unlimited numbers of identical somatic embryos, and ultimately trees may be obtained. Synthetic seed technology is unlikely to replace conventional breeding strategies, but the two techniques can be used to complement each other. In addition, somatic embryogenesis can be used to extend our fundamental knowledge of the process in conifers (Attree and Fowke, 1991).

Somatic versus zygotic embryogenesis

The development of somatic embryos closely resembles that of zygotic embryos both morphologically and temporally. They are bipolar structures presenting shoot and root meristems, with a closed independent vascular system and, frequently single-cell origin and production of specific proteins. The similarity between zygotic and somatic embryogenesis is both striking and remarkable, considering that somatic embryos develop completely outside both the physical constraints and the informational context of maternal tissue. The fact that structurally and developmentally normal embryos can develop from somatic cells demonstrates that the genetic program for embryogenesis and its elaboration are totally contained within the cell and can function completely in the absence of gene products from the maternal environment. Although the hormonal content of growth medium may somehow mimic some natural signal to initiate embryogenesis, it is clear that the morphology and size of the various embryo stages are completely intrinsic in the embryogenic program and are not controlled by any

environmental information or spatial limitations (Zimmerman, 1993). With regard to zygotic embryogenesis, the development of the embryo passes through the octant, globular, oblong, heart, torpedo, and cotyledonary stages and eventually to the mature dehydrated embryo. Three major events take place: (1) the first asymmetric division of the zygote, giving a small apical cell that generates the embryo and a large basal cell which will form the suspensor (*i.e.* a specialized structure that facilitates continued development of the embryo proper within the seed), (2) specific pattern formation, which takes place in the globular embryo, (3) the transition to the cotyledonary stage which coincides with the initiation of the root primordium followed, in dicots, by the shoot primordium. The zygote is intrinsically embryogenic which is the opposite of somatic embryogenesis. The latter requires the induction of embryogenic competence in cells which are not naturally embryogenic (Dodeman *et al.*, 1997). In addition, somatic embryos lack of differentiation of endosperm and suspensor tissue and do not experience desiccation and dormancy, but rather continue to grow into fully differentiated plantlets (Zimmerman, 1993; Fehér *et al.*, 2003). Overall, somatic embryos mimic their zygotic counterparts in morphological, developmental and molecular aspects, and protein composition. In the study by Winkelmann *et al.* (2006), a systematic comparison of the proteomes of zygotic and somatic embryos has been reported in *Cyclamen persicum*. Relatively few proteins were present in different relative abundance if somatic embryos were compared to zygotic embryos. Some enzymes involved in glycolysis were specifically induced in somatic embryos. Nonetheless, their higher abundance was evoked by the exogenous sucrose supply by tissue culture media which for somatic embryos is at higher concentration than that for zygotic embryos. Other proteins occurring in high concentrations in cyclamen somatic embryos were heat shock 70 proteins which are formed in response to stress and also are developmentally regulated. Some globulins, the major seed storage proteins in dicotyledonous plants, were found in somatic embryos and in lower amounts in zygotic embryos (Winkelmann *et al.*, 2006). Savona *et al.* (2012) isolated two genes, *C. persicum somatic embryogenesis receptor-like kinase 1* and *2* (*CpSERK1* and *CpSERK2*), encoding leuchine-rich repeat receptor-like kinases, from cyclamen embryogenic callus. They reported that *CpSERK1* and *CpSERK2* gene expression is high in embryogenic, moderate in organogenic, and null in recalcitrant calli. Similar patterns of expression of these two genes occurred in zygotic and mature somatic

embryos. Indeed, CpSERK1 and CpSERK2 proteins are involved in defining and maintaining meristems in both somatic and zygotic embryos (Savona *et al.*, 2012).

Physiological phases of somatic embryogenesis process and embryogenic competence

Somatic embryos can differentiate either directly from explant tissue without an intervening callus phase or indirectly through callus tissue. Direct embryogenesis takes place from embryogenically predetermined cells which are able to undergo embryogenesis without dedifferentiation, *i.e.* callus formation. In contrast, indirect somatic embryogenesis takes place from undetermined cells and non-differentiated callus should first be formed (von Arnold *et al.*, 2002; Gaj *et al.*, 2004). In the latter case, a more complex medium with additional factors is required to induce dedifferentiation and initiation of cell division in the explant before they can express embryogenic competence (Namasivayam, 2007). The callus formed may be either embryogenic or not. It is usually easy to distinguish between embryogenic and non-embryogenic callus on the bases of its morphology and colour. Embryogenic callus is composed of proembryogenic masses. It is not known if the first formed proembryogenic masse is actually an embryo which deviates from the normal embryo development and proliferates in response to plant growth regulators (von Arnold *et al.*, 2002). Indeed, somatic embryogenesis might be also induced directly, or through callus, in the culture of somatic embryos, and this process is called secondary somatic embryogenesis in contrast to primary somatic embryogenesis induced from explants cells. Nonetheless, the most frequent mode of embryogenesis is *via* callus formation, which is an indirect type of regeneration (Gaj *et al.*, 2004). During the initial phases of embryogenesis process, somatic cells must embark on a progression of developmental events referred to as dedifferentiation, competence acquisition, induction and determination (Thomas *et al.*, 2004). Other authors suggested that differentiated somatic cells acquire embryogenic competence during the induction phase. According to Namasivayam (2007), the somatic embryogenesis process can be divided into two phases: induction and expression. In the expression phase, the embryogenic cells display their embryogenic competence and differentiate to form somatic embryos. The two phases were suggested to be independent of each other and influenced by different factors. The term ‘embryogenic cells’ is restricted to those cells that have completed their transition from a somatic state to one in which further exogenous stimuli, such as the application of

growth regulators, are necessary to produce the somatic embryo. The cells in a transitional state that still require only minimal exogenously applied stimuli to become embryogenic are defined as competent cells. It is still unclear what changes a somatic cell has to undergo in order to become an embryogenic cell and capable of forming an embryo at a later stage of development. In general, an embryoid may arise from a single cell, or a group of cells 'budding', depending on neighbour relationships of cells within the explant. The conditions for the induction of somatic embryogenesis in different species and cultivars are usually discovered by trial and error by analyzing effects of different culture conditions such as: plant growth regulator balance, osmotic conditions, changing pH, amino acid and salt concentrations, heat shock and treatment with various chemical substances (Namasivayam, 2007). Based on histological observations, the embryogenic cells that form somatic embryos are characterized generally as small, isodiametric in shape; they have large and densely staining nuclei and nucleoli, and are densely cytoplasmic. It is not at all clear how the embryogenic cells originate and whether the differential ability of somatic cells to become embryogenic reflects differential ability of different genotypes or of different cell types within the same genotype. Competent cells have a variable appearance that prevents their identification on the basis of morphology. Studies on carrot and alfalfa cultures suggested that cell polarity and an asymmetric first cell division are involved in the initiation of somatic embryogenesis. Exogenous growth regulators probably modify the cell polarity by interfering with pH gradients or electrical fields around the cells. However, members of the heterogenous embryogenic carrot cell population were capable of forming somatic embryos after either apparently equal or unequal first cell division. This implies that the correct plane of division is not necessary for somatic embryogenesis. However, asymmetrical distribution of intracellular molecules or cell constituents correlates with differential cell fate. Several studies have showed that changes in intracellular pH may also contribute to the acquisition of embryogenic competence. Another feature for the acquisition of the embryogenic competence is physical isolation of the cell from others such as absence of plasmodesmatal contact with the neighbouring cells in embryogenic culture (Namasivayam, 2007). For this, changes in the cell wall composition throught callose (1,3-β-glucan) deposition between the plasma membrane and the wall of competent cells are thought to occur shortly after the induction treatment (You *et al.*, 2006). The extracellular matrix was reported to be

present on the surface of embryogenic tissues, but not in the non-embryogenic tissues. The extracellular matrix layer was found to be dominant in the pre-embryogenic stage and reduced to fragments during embryo growth and development in mature embryogenic tissue. These studies suggest that isolation may be required to allow induction of new morphogenetic events and to prevent interference from adjacent tissues which are degenerating or committed to different pathways (Namasivayam, 2007).

The role of auxins in somatic embryogenesis

Auxins are known to have a crucial role in the induction of somatic embryogenesis in tissue cultures. However, once the induction period is accomplished, the process generally continues on medium lacking auxin since a high concentration in the medium might inhibit somatic embryo development (Thomas *et al.*, 2002). The synthetic auxin 2,4-D is commonly used to initiate tissue cultures of several species. The presence of 2,4-D in the culture medium often resulted in undifferentiated callus proliferation. However, either the removal or lower concentration of 2,4-D from/in the growth medium of cultured, undifferentiated cell clusters could commence an organized growth pattern and develop into somatic embryos (Ribnicky *et al.*, 1996). It has been also reported that 2,4-D could inhibit the embryogenic potential in *Citrus*. Cultures of stigma and style transverse thin cell layer from *C. limon* and *C. sinensis* exposed to 2,4-D never differentiated embryos, whereas they did on growth regulator free medium (Fiore *et al.*, 2002). NAA has been demonstrated to be an effective auxin for both the initiation and maturation of somatic embryos in carrot. Not only is the exogenous auxin important in the initiation of somatic embryogenesis, but changes in endogenous auxin concentrations may also be critical for the later stages of embryogenesis as well. It has been shown that embryogenic carrot cells grown in the presence of 2,4-D contain large amounts of IAA. The transfer of embryogenic cell clusters to medium lacking 2,4-D, which initiated the process of somatic embryo formation, was shown to be coincident with a large decrease in endogenous IAA. A high IAA concentration may be required to induce the competence of cells to undergo somatic embryogenesis, and the subsequent decrease of IAA may be required for the development of the somatic embryos that follows (Ribnicky *et al.*, 1996). Ribnicky *et al.* (1996) studied the effect of exogenous auxins on endogenous IAA metabolism by culturing excised carrot hypocotyls both in the absence of exogenous auxin and in the

presence of 2,4-D, IAA or NAA. They observed that all of the exogenous auxins accumulated in large quantities, suggesting that auxin uptake into plant material is an efficient process. Treatment with 2,4-D had only minor effects on the concentration of endogenous IAA, indicating that 2,4-D may act directly in carrot hypocotyls and not indirectly through induction of changes in endogenous IAA concentration. Of the auxins tested, 2,4-D accumulated to the highest concentration. The slow formation of conjugates of 2,4-D contrasts with the more rapid conjugation found with IAA and NAA, suggesting that the processes are very different. The effects of 2,4-D in tissue culture seem to require high concentrations of the growth regulator. If 2,4-D is removed from tissue culture growth medium, the growth effects of the 2,4-D are lost even though the plant materials contain significant quantities of 2,4-D. NAA accumulated in much lower total amounts than 2,4-D, and the amount of free NAA was much less than the amount of conjugated NAA. Nevertheless, the concentration of free NAA was significantly higher than the concentration of free IAA in untreated cultures, and, therefore, the free NAA seemed directly responsible for the observed auxin-like changes in the growth of these cultures. NAA, like IAA, has been shown to induce its own conjugation when applied to plants. Cultured carrot hypocotyls conjugated NAA much more readily than they did in the presence of 2,4-D. In terms of accumulation and conjugation, NAA appears to be intermediate between IAA and 2,4-D. The developmental effects of NAA treatment were also intermediate between those of 2,4-D and IAA (or no auxin treatment). NAA-treated hypocotyls produced callus and became organogenic, producing roots, shoots, and embryos. In light of these findings, the developmental effects of treatment with 2,4-D and NAA are most likely due to their ability to act directly as auxins and not due to their ability to perturb IAA metabolism. The very limited callus growth observed in IAA-treated carrot hypocotyls supports the hypothesis that the conjugation of IAA reduces its activity. Exogenous IAA activated the IAA conjugation system, which moderated the internal concentration of the free hormone. In addition, IAA has a negative influence on *de novo* IAA biosynthesis, suggesting that IAA activates a feedback inhibition of IAA biosynthesis. Moreover, since IAA is degraded by both light and nutrient salts, the lack of significant responses to the IAA treatment has been attributed to its chemical lability (Ribnicky *et al.*, 1996). In addition, it has been suggested that the polar auxin transport in early globular embryos is essential for the establishment of bilateral symmetry during plant

embryogenesis. For the induction of the process leading to polarity, relatively high levels of endogenous, free IAA may be necessary. However, once induction has occurred, those high levels of IAA must be reduced to allow the establishment of the auxin gradient. If the levels are too low or high or do not diminish after the induction, the gradient cannot be formed and thus somatic embryogenesis cannot progress (Deo *et al.*, 2010).

The role of cytokinins in somatic embryogenesis

Although somatic embryo induction usually involves the use of auxins, mostly 2,4-D, with or without a low level of cytokinin, initiation of somatic embryos on medium containing cytokinin as the sole growth regulator has been reported in few species. Carimi *et al.* (1995; 1999) reported that BAP was able to stimulate callus formation and embryogenic response in both styles and transverse thin cell layer of pistil from different species of *Citrus*. Cytokinins were used to induce somatic embryo formation in tuber-derived callus of *Corydalis yanhusuo*, a medicinal plant belonging to Papaveraceae. The cytokinin efficiency varied depending on the cytokinin tested. Although kinetin and BAP induced the highest number of somatic embryos, the embryos induced on these cytokinins reverted to callus. The embryos developed on zeatin containing medium, instead, progressed through the globular, late-globular, heart, early cotyledonary and cotyledonary stages (Sagare *et al.*, 2000). Embryo formation on leaf explants of *Oncidium*, a sympodial orchid, was retarded by auxins, whereas it was promoted by cytokinins (Chen and Chang, 2001). In sunflower, the presence of BAP and a high concentration of sucrose in the culture media are an absolute requirement to induce cell divisions specifically involved in the direct regeneration of somatic embryos in immature zygotic embryos (Thomas *et al.*, 2002; 2004).

Embryogenic activity of urea derivatives

The potential of urea derivatives in regulation of somatic embryogenesis has been revealed in several studies. Somatic embryogenesis was induced in hypocotyl explants of geranium cultured on media supplemented with various concentrations of TDZ. In less than two weeks, somatic embryos were observed in treatments containing TDZ. The use of BAP in combination with IAA also evoked embryogenesis, but the efficiency of somatic embryo production was significantly lower than that obtained with TDZ. The use of TDZ

resulted in an approximately twofold increase in somatic embryo production over that achieved with BAP plus IAA. The use of BAP alone caused swelling of the explants, but supported only an occasional appearance of somatic embryos (Visser *et al.*, 1992). Chen and Chang (2001) reported that among the cytokinins tested on somatic embryogenesis on leaf explants of *Oncidium*, TDZ was found to be the most effective in inducing the greatest percentages of embryo formation and the highest number of embryos per explant. The synthetic cytokinin 4-CPPU induced callus formation and somatic embryogenesis in stigma and style transverse thin cell layer of *Citrus limon* and *C. sinensis* (Fiore *et al.*, 2002). More recently, Carra and colleagues (2006) investigated the effect of three urea derivatives, PBU, 2,3-MDPU and 3,4-MDPU, on embryogenic competence of three *Citrus* species. The three compounds influenced the production of somatic embryos differently with regard to the concentration tested and the *Citrus* species. The best results were obtained when stigma and style explants of *C. madurensis* were cultivated in the presence of the highest concentration of 2,3-MDPU, when the same explants of *C. limon* were cultivated in the presence of the intermediate concentration of PBU or in the presence of the highest concentration of 3,4-MDPU, or when the same explants of *C. myrtifolia* were cultivated in the presence of the intermediate or the highest concentration of PBU. All three compounds showed higher embryogenic performance when compared with adenine-type cytokinin (BAP) or urea-type cytokinin (4-CPPU).

Other factors influencing somatic embryogenesis

In vitro development of cells and tissues depends on different factors, such as: genotype, type of plant, age and developmental stage of an explant, physiological state of an explant-donor plant, and the external environment which includes composition of media and physical culture conditions (light, temperature). Interaction between all these factors leads to the induction and expression of a specific mode of cell differentiation and development (Gaj *et al.*, 2004). Initiating the developmental program for somatic embryogenesis from a cell or a group of cells frequently depends on the nature of the explant source. This refers to the conditions under which the source plant was grown and the stage of development of the plant part from which the explant was taken. Juvenile tissues appear to be the most suitable for induction of somatic embryogenesis. Somatic embryogenesis can be induced in cultures of various explants: anthers, pollen, ovaries,

roots, leaves, petioles and stems, immature and mature embryos, mature cotyledons and corms (Gaj *et al.*, 2004; Deo *et al.*, 2010). It has become widely recognized that somatic cells can acquire embryogenic potential as a result of dramatic changes in the cellular environment. Mechanical wounding of explants, high salt concentration, heavy metal ions or osmotic pressure, starvation, pH, low or high temperature, hormone supply, generally called stress factors, positively influenced somatic embryo induction in diverse plant species (Fehér *et al.*, 2003; Gaj *et al.*, 2004). The response to stress conditions depends on two main parameters: the level of stress and physiological state of the cells. If the stress level exceeds cellular tolerance, the cells die. In contrast, low level of stress enhance metabolism and induce adaptation mechanisms. Adaptations include the reprogramming of gene expression, as well as changes in the physiology and metabolism of the cells. This transient cell state induced by stress conditions can be characterized by extensive cellular reorganization and, if appropriate signals are perceived, a developmental switch is allowed. Dedifferentiation, in many cases, can be clearly correlated with stress and/or auxin responses of cells (Fehér *et al.*, 2003). Hormones are the most likely candidates in the regulation of developmental switches. Although auxins and cytokinins are the main growth regulators in plants involved in the regulation of cell division and differentiation, other plant growth regulators and biologically active molecules other than plant growth regulators have been reported to induce somatic embryogenesis in different plant species. For instance, it has been reported that somatic embryos synthesize and accumulate ABA. Although ABA hormonally regulates desiccation-dormancy program of zygotic embryos, the application of ABA has a positive effect in inducing somatic embryos formation and maturation in several plant species (Zimmerman, 1993). Fernando and Gamage (2000) hypothesized that the positive effect of ABA observed in somatic embryo formation from immature zygotic embryo-derived callus of coconut could be a result of increased production of storage reserves and uptake of sucrose from the medium. Exogenously supplied ABA was an important component of maturation medium for hybrid larch somatic embryos. In the absence of ABA, maturation resulted in poorly developed somatic embryos exhibiting abnormal morphology. In contrast, the presence of ABA promoted the development of higher quality somatic embryos in large quantities (Gutmann *et al.*, 1996). Certain bioactive compounds such as amino acids, glutamine, proline and tryptophan and polyamines such as putrescine have been identified as

enhancers of somatic embryogenesis in some species (Deo *et al.*, 2010). Modulation of polyamine metabolism has been studied in different systems in relation to somatic embryogenesis, demonstrating that polyamines are crucial endogenous factors during *in vitro* embryo formation. Bertoldi *et al.* (2004) reported that polyamines are involved in somatic embryogenesis in *Vitis vinifera* callus cultures. These authors suggested that these compounds could be involved in the active cell division taking place during embryo differentiation, rather than in the cell extension predominant in developing plants. Sugars are included in all tissue culture media as an essential source of energy, and create the appropriate osmotic conditions for *in vitro* cell growth. The type and concentration of sugar used in media influences somatic embryogenesis. Sucrose has been most frequently employed to induce somatic embryogenesis in different plant species. Rarely, other sugars have been found useful for somatic embryogenesis induction. In some protocols, supplementation of the media with more than one type of sugar was recommended to increase somatic embryogenesis efficiency. The concentration of sugar in an induction medium can be a key factor in switching on/off the specific type of *in vitro* morphogenic pathway. Sucrose concentration had been found to directly influence the uptake of BAP into the sunflower explants, and shortly later endogenous auxins and cytokinin level was modified which in turn triggered organogenic or embryogenic response (Gaj *et al.*, 2004). Light is one of the most important environmental signals. Reduced light intensity or darkness might be required to induce somatic embryogenesis because this will minimize the production of inhibitory compounds from tissues in the culture medium (Deo *et al.*, 2010).

Somatic embryogenesis in Medicago truncatula

Legumes are protein-rich plants widely cultivated for food and forage. The growth of legumes is independent of an external supplement of reduced nitrogen, because of the fixation of dinitrogen in symbiosis with rhizobia. The ecological and agricultural importance of this process has fuelled much of the incentive to study plant-microbe relations. The development of nitrogen-fixing symbiosis is a complex process determined by both partners. One of the most extensively characterized systems, from the viewpoint of the bacterial partner, is the symbiosis between *Rhizobium meliloti* and *Medicago sativa*. However, the perennial cultivated alfalfa is a tetraploid and allogamous with varying

degrees of self-incompatibility and it has a large genome. These drawbacks have led to adopt a diploid autogamous annual *Medicago* species, *M. truncatula* Gaertn. ecotype Jemalong, as a nominal model plant for genetic and molecular analyses of symbiosis. However, Jemalong shows limitations in nitrogen-fixing symbiosis with some *R. meliloti* strains. Hoffman *et al.* (1997) isolated a new *M. truncatula* line suitable for genetic analysis of *Medicago*-*R. meliloti* symbiosis because of its high regeneration capacity and broad *R. meliloti* strain specificity. This line, namely R108, has a greatly enhanced or *de novo* capacity to form somatic embryos and can be efficiently regenerated into diploid fertile plants. To evaluate the regeneration capacity of this line, different types of explants derived from *M. truncatula* line R108 were incubated in proembryogenic callus induction medium containing 2,4-D and BAP. Best results were obtained when petiole and leaf explants were used (Hoffmann *et al.*, 1997). In the following study, the effect of 2,4-D and BAP on embryo development and regeneration of leaf explants of R108 was investigated (Trinh *et al.*, 1998). Using the medium containing both 2,4-D and BAP, tenfold more plantlets per explant were obtained than by using the same medium containing 2,4-D only. Afterwards, the same authors studied the influence of the physiological stages of the donor plant of R108 line on embryogenesis and regeneration (Kamaté *et al.*, 2000). They reported that leaf explants from 3- to 7-week-old donor plants required only 4 weeks for callus formation, whereas those from 9- to 24-week-old plants took 6-7 weeks. In addition, they observed that the number of embryos per explant decreased strongly with the age of the donor plant.

Shoot regeneration

In plants, particularly during *in vitro* culture, somatic cells may be capable of regeneration: when given the proper stimuli they may form adventitious roots, shoots or embryos. Biotechnological breeding and propagation methods depend on this (reviewed in de Klerk *et al.*, 1997). While embryogenesis is usually postulated as initiated from a single cell, organogenesis should involve several initial cells (Monacelli *et al.*, 1988). In contrast to the embryogenic pathway, the *de novo* organogenetic pathway is more often used in biotechnological breeding methods (*i.e. in vitro* micropropagation, haploid production

and genetic engineering), particularly in dicotyledonous plants, mainly because the plant explants and *in vitro* conditions are relatively simple and more robust (reviewed in Duclercq *et al.*, 2011). Excised plant tissues that have lost an apical meristem have the ability to regenerate new root and/or shoot apical meristems in the absence of sexual fertilization. If an explant gives rise to a root- of shoot-forming apical meristem without an intervening embryo phase, the process is termed somatic organogenesis. Somatic organogenesis is critical to the survival of many species in nature, but can also be induced *in vitro* using exogenous phytohormones (Nameth *et al.*, 2013). The *in vitro* morphogenic responses of cultured plant tissues are affected by several factors such as ecotype, explant tissue, age of the donor plants, light, temperature, pH and composition of the culture medium (Gubiš *et al.*, 2004). In pioneering experiments, Skoog and Miller (1957) demonstrated that the developmental fate of regenerating tobacco pith cells in tissue culture could be directed by the balance of cytokinin and auxin added to the growth medium: high cytokinin/auxin ratios promoted shoot formation, and low ratios favoured the formation of roots. Christianson and Warnick (1983) described shoot regeneration in tissue culture in developmental terms. They divided the process in *Convolvulus* leaf explants into three phases, competence acquisition, induction, and differentiation, based on the changing hormone requirements for each phase. During the first phase, cells 'de-differentiate' and proliferate to produce callus. During this phase, the explanted tissue acquired the competence to respond to inductive signals. In the next phase, the induction phase, cells are responsive to the organogenic stimulus and become determined to form specific organ, *i.e.* a shoot. Only during this phase the hormonal composition of the medium is critical. In the third phase, the newly determined tissue forms a functional meristem and develops into a complete shoot independent of exogenous hormones. The succession of steps during regeneration is very similar for root-, shoot- and embryo formation. The first stage may involve a period of callus growth (indirect regeneration). Often, though, competent cells already exist in the explant and respond to the organogenic/embryogenic stimulus after a (short) lag phase without any cell division or without cell division at a large scale (direct regeneration) (reviewed in de Klerk *et al.*, 1997). Nonetheless, *in vitro* regeneration of most important crop plants does not strictly follow these stages. As a consequence, the development of predictable and routine shoot organogenesis remains a challenge. In addition, each developmental stage is

characterised by the expression of a specific set of genes, from the initial phytohormone response to late-shoot meristem development. However, to date no clear picture of the gene regulatory network associated with shoot organogenesis in cultured explants is available. Particularly, the precise molecular mechanisms determining 'when' and 'where' shoot organogenesis (*e.g.* neoformation of meristem in the callus) is going to happen are not known (reviewed in Duclercq *et al.*, 2011). In *Arabidopsis*, callus formation can be stimulated by an initial low dose of cytokinin to trigger cell division along with a high dose of the synthetic auxin 2,4-D. To stimulate subsequent shoot organogenesis, high levels of cytokinin are used along with low levels of NAA (Nameth *et al.*, 2013).
Plants regenerated from *in vitro* cultures of somatic cells very frequently differ from the original phenotype as a result of uncontrolled and spontaneous genotypic variation originating during cell and tissue culture. This phenomenon, which has been defined 'somaclonal variation' (Larkin and Scowcroft, 1981), is confined to plants regenerated from dedifferentiated cells. Somaclonal variation might be undesired when there is a clear expectation of genetically uniform plants. On the other hand, genetic uniformity can often be exploited for commercial purposes since somaclonal variation can genetically improve plants via nonsexual methods (Monacelli *et al.*, 1988; Sarwar and Skivin, 1997).

Shoot regeneration in Lycopersicon esculentum

Tomato (*Lycopersicon esculentum* Mill.) is one of the most studied higher plants because of its importance as a crop species, and of several advantages for genetic, molecular and physiological studies. Shoot formation from explants of cotyledons, stems, petioles, leaves, anthers and inflorescence has been reported in tomato (Gubiš *et al.*, 2004). Monacelli *et al.* (1988) determined the developmental pattern of tomato shoots from *in vitro* cultured cotyledonary explants of tomato in the presence of both IAA and ZR. They reported that callus growth occurred exclusively at the sites of excision and shoots differentiated from these. Differentiation was by organogenesis, while embryogenesis was never observed. Callus formation and subsequent shoot differentiation showed polarity, being consistently more abundant at the proximal excision site of each explant compared to the distal site. Shoot differentiation occurred asynchronously, starting on day 5 of culture with the division of a few cells of a neocambial strand differentiated within the superficial region of the callus. Divisions of 2-4 initial cells led to the formation

of zones of intense meristematic activity from which shoots and glandular hairs differentiated. Shoots were formed by the development of a vegetative dome. The external cells of each dome divided anticlinally, producing a cellular layer which then becomes the tunica of the future apex (Monacelli *et al.*, 1988). *In vitro* shoot regeneration from tomato cotyledons has been adopted as a regeneration test to evaluate the effectiveness of new synthetic compounds in several studies (Branca *et al.*, 1990; Ricci *et al.*, 2001a; 2001b; 2006). Branca *et al.* (1990) reported that two synthetic auxins, such as 1,2-benzisoxazole-3-acetic acid (BOAA) and 1,2-benzisothiazole-3-acetic acid (BIA), when simultaneously supplied with ZR, showed different activity on tomato organogenesis, being BOAA more effective in inducing shoot formation than BIA. Ricci *et al.* (2001b; 2006) studied the hypothetical cytokinin-like activity of MDPUs and BDPUs, alone or simultaneously supplied with BOAA, using this experimental test. Neither MDPUs nor BDPUs were able to induce shoot regeneration both alone and in the presence of BOAA.

AIM OF THIS STUDY

In the research by Ricci *et al.* on chemical structure-biological activity relationship, two symmetric urea derivatives, namely *N*,*N*'-bis-(2,3-methylenedioxyphenyl)urea (2,3-MDPU) and the *N*,*N*'-bis-(3,4-methylenedioxyphenyl)urea (3,4-MDPU), have been identified for their ability to enhance adventitious root formation, without showing any biological activity *per se* (Ricci *et al.*, 2001b; 2003; 2008). Indeed, even if structurally very similar to ureic-type cytokinins, MDPUs did not show any cytokinin-like activity. Their possible auxin-like activity was also excluded by testing them as auxins in different bioassays. Instead, their ability to promote rooting was ascribed to the interaction with endogenous or exogenous auxin without being auxin synergist. Furthermore, when used to induce somatic embryogenesis in *Citrus*, MDPUs showed high embryogenic performance (Carra *et al.*, 2006). When a chemical modification of the aromatic moiety of MDPUs consisting in a replacement of the methylenedioxyphenyl groups by the isostere benzoxazole ones was performed, two new compounds, 1,3-di(benzo[*d*]oxazol-5-yl)urea (5-BDPU) and the 1,3-di(benzo[*d*]oxazol-6-yl)urea (6-BDPU), were obtained. BDPUs showed a conserved adventitious rooting enhancement, without either auxin- or cytokinin-like activity *per se*, similarly to the lead compounds (Ricci *et al.*, 2006). Starting from the previously obtained results, the aim of this study was either to investigate the action spectrum of the BDPUs or to understand their mode of action, trying to answer to these questions:

- Is the BDPU rooting adjuvant activity clearly evident in 'model plants' only, or is it quite ubiquitous in Gymnosperms and Angiosperms, in herbaceous and woody species?
- Do BDPUs affect the auxin local pools at the level of sensitive cells capable to respond to auxin stimulus?
- Is the adventitious rooting adjuvant activity shown by BDPUs independent from the type/chemical structure of the exogenous auxins used to induce adventitious roots?

- Do BDPUs interact with auxin signalling pathway during the adventitious root formation?
- Do BDPUs interact with cytokinin signalling pathway during the adventitious root formation?
- Is their adjuvant activity limited to the adventitious rooting process or do they exert a similar behaviour in other physiological processes, in which auxins and/or cytokinins are involved, as shoot regeneration and somatic embryogenesis?

The steps of this study are:

- Verify the cooperation of BDPUs 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
- Analyse the effect of BDPUs on the adventitious rooting capacity of difficult to root carob and strawberry tree microcuttings in response to IBA and mixture of IBA and BDPUs
- Determine the time course of cellular events leading to the formation of adventitious roots in response to IBA and mixture of IBA and BDPUs through histological analyses of *Pinus* and carob tissues
- Understand if and how BDPUs affect the auxin local pools at the wounded site of *Pinus* cuttings by analysing the spatial distribution of endogenous IAA in the presence of IBA and mixture of IBA and BDPUs
- Evaluate specific sites and patterns of auxin distribution through histochemical staining of GUS activity in *DR5-GUS* transgenic *Arabidopsis* seedlings in response to auxins, IBA and NAA, and mixture of IBA or NAA and BDPUs
- Analyse the interaction of BDPUs with the auxin signalling pathway by evaluating the expression of two genes, *PrSCL1* and *PrSHR* in response to IBA and mixture of IBA and BDPUs

- Analyse the hypothetical interaction of BDPUs with the cytokinin signalling pathway through CRE1/AHK4 receptor in response to cytokinins and mixture of cytokinins and BDPUs
- Evaluate specific sites and patterns of an early cytokinin response gene expression through histochemical staining of GUS activity in *ARR5-GUS* transgenic *Arabidopsis* seedlings in response to cytokinins and mixture of cytokinins and BDPUs
- Assess the effect of mixture made by BDPUs and different types of cytokinins in shoot regeneration bioassay of *Lycopersicon esculentum* explants
- Investigate the effect of BDPUs to induce the production of somatic embryos in *Medicago truncatula* line R 108 explants in the presence of mixture of auxin or cytokinin and BDPUs

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), the 1-naphtalene acetic acid (NAA) and the *N*-phenyl-*N*'-(1,2,3-thiadiazol-5-yl)urea (TDZ) were dissolved in dimethylsulfoxide (DMSO). The final concentration of DMSO in the *in vitro* culture medium or in the aqueous solutions did not exceed 0.2% (Schmitz and Skoog, 1970). The 5-BDPU and the 6-BDPU were synthesized by the Pharmaceutical Department of the University of Parma, as previously reported (Ricci *et al.*, 2006). The N^6-(2-Isopentenyl)adenine (2iP), the N^6-benziladenine (BAP), the indole-3-butyric acid (IBA) were dissolved in distilled water and then sterilized using 0.2 µM sterile disposable filter units. The zeatin riboside (ZR) was dissolved in 70% ethanol. The 1,2-benzisoxazole-3-acetic acid (BOAA), utilized as auxin for tomato regeneration test (Ricci *et al.*, 2001a), was dissolved in distilled water and then sterilized using 0.2 µM sterile disposable filter units.

Adventitious rooting

- Adventitious rooting of *Arabidopsis thaliana* etiolated seedlings

Plant material and growth conditions

Seeds of *Arabidopsis thaliana* ecotype Columbia (Col-0) were sterilized by soaking in 70% (v/v) ethanol for 1 minute and 50% (v/v) commercial bleach (equivalent to 2.5% NaOCl) for 10 minutes and then washed 5 times with sterile distilled water. Seeds were sown in glass culture pots containing ¼ MS nutrients (MS salts) supplemented with 0.8% (w/v) agar. pH was adjusted to 5.8 with 0.1 M NaOH before autoclaving. Pots were capped with aluminium foil and grown in a growth chamber at 26 ± 1 °C. Three-day-old etiolated seedlings were used as explants for the rooting experiments.

Adventitious root induction

Three-day-old etiolated seedlings were plated in Petri dishes containing MS medium (MS salts and vitamins), 3% (w/v) sucrose, 1.5% (w/v) agar. pH was adjusted to 5.8 with 0.1 M

NaOH before autoclaving. For adventitious root induction, the medium culture was supplemented with 5-BDPU or 6-BDPU alone, IBA or NAA as exogenous auxins or with mixtures of 5- or 6-BDPU and IBA or NAA (for compound concentrations and rooting treatments of *Arabidopsis* seedlings, see the **table 1** below). Medium containing only DMSO was used as control. Plates were placed in racks in a vertical orientation under 16-h day length at 26 ± 1 °C, with light provided by cool-white fluorescent lights at a photon flux of approximately 27 µmol m^{-2} s^{-1} for 7 days. Emergent adventitious roots were quantified on the 7th day using a stereomicroscope. Results were evaluated in terms of mean number of adventitious roots per rooted seedling. Each treatment consisted of three replicates of 10 etiolated seedlings. The experiments were repeated twice.

Table 1 ◘ Rooting conditions of *Arabidopsis thaliana* three-day-old etiolated seedlings:
- DMSO
- 1, 2, 4 or 8 µM 5-BDPU or 6-BDPU
- 0.1 µM IBA or NAA
- 0.1 µM IBA + 1, 2, 4 or 8 µM 5-BDPU or 6-BDPU
- 0.1 µM NAA + 1, 2, 4 or 8 µM 5-BDPU or 6-BDPU

Arabidopsis seedlings were exposed to the indicated treatments for the entire rooting period (7 days).

Statistical analysis

Significantly different means ($p<0.05$) were identified by using Student's *t* test (SPSS Statistic 17.0).

- Adventitious rooting of *Malus pumila* stem slices

Plant material and growth conditions

Microcuttings of *Malus pumila* Mill. rootstock M26 were deprived of apices and propagated on a micropropagation medium (MS salts plus 0.4 mg l^{-1} thiamine HCl, 0.5 mg l^{-1} nicotinic acid, 0.5 mg l^{-1} pyridoxine HCl, 0.1% (w/v) myo-inositol, 1% (w/v) sucrose, 2% (w/v) sorbitol, 0.8% (w/v) agar, pH 5.8). The medium was supplemented with 1.3 µM BAP and 0.25 µM IBA. After 5 weeks culture at 26 ± 1 °C at light intensity of 27 µmol m^{-2} s^{-1} under 16-h day length, the new developed shoots 2-2.5 cm in length were separated and

used for further multiplication or for preparing stem slices.

Adventitious root induction

The stem slice system was performed according to Van der Krieken *et al.* (1993). One-mm stem slices were cut from defoliated shoots with a scalpel blade. Groups of 16 slices were incubated in Petri dishes with the apical side on a nylon mesh put on the micropropagation medium supplemented with NAA alone or plus 5-BDPU (for compound concentrations and rooting treatments, see **table 2**). Adjacent slices from the same shoot were distributed over different rooting treatments, to prevent their weak rooting capacity correlation (de Klerk and Caillat, 1994). Plates were incubated upside down in the dark to avoid auxin degradation at 26 ± 1 °C for 6 days. Then, the nylon mesh with the slices attached was transferred to a Petri dish containing *hormone-free* medium. Dishes were then incubated at 26 ± 1 °C under 16-h photoperiod with a light intensity of 27 µmol m^{-2} s^{-1} provided by cool-white fluorescent lights for 8 days. The number of rooted slices was counted after 14 days from the beginning of the rooting experiment. Experiments were carried out in triplicate and repeated twice.

Table 2 ◘ Rooting treatments of *M. pumila* rootstock M26 stem slices:

- 1 µM NAA
- 1, 2, 4 or 8 µM 5-BDPU
- 1 µM NAA + 1, 2, 4 or 8 µM 5-BDPU

Apple stem slices were exposed to the indicated treatments for 6 days. Explants were then transferred and kept in HF medium until 14th day from the beginning of the rooting experiment.

- Adventitious rooting of *Pinus radiata* hypocotyl cuttings

Plant materials and growth conditions

Pinus radiata D. Don seeds were provided by Oihanberri (Vitoria, Spain). Seeds were kept at room temperature. They were stratified by soaking them in running tap water at room temperature for 12 h and then sown in fine wet vermiculite. Seeds were then covered with a fine layer of dry vermiculite. Seedlings were grown with a light regime of 8 h darkness at 21 °C and 16 h light provided by cool-white fluorescent lights (27 µmol m^{-2} s^{-1}) at 25 °C and moistened with distilled water weekly.

Adventitious root induction

Hypocotyl cuttings were prepared from seedlings 21 days after germination. The primary root was cut off from the seedling at 2.5 cm below the cotyledons with a razor blade. Thus, the cut was above the hypocotyl/root junction. Groups of five cuttings were placed in 20-ml vials containing aqueous solutions of either IBA or NAA alone or in the simultaneous presence of 5-BDPU or 6-BDPU (for compound concentrations and rooting treatments see **table 3** below). The entire portion of the hypocotyl, but not the cotiledons, was submerged in the solution. Cuttings treated with distilled water or with solutions of BDPUs alone were used as controls. Vials were then returned to the same growth chamber used for seedling growth and refilled daily with distilled water. Four weeks after, the number of roots or root primordia visible externally was recorded for each cutting. One vial was considered as one observation. All the experiments were carried out in triplicate and repeated twice. Results were expressed as rooting percentage and mean number of adventitious roots per rooted cuttings.

Table 3 ◘ Rooting conditions for *Pinus* cuttings:
- distilled water
- 0.1, 1 or 10 µM IBA
- 0.1, 1 or 10 µM NAA
- 0.1, 1 or 10 µM 5-BDPU or 6-BDPU
- 0.1, 1 or 10 µM IBA + 0.1, 1 or 10 µM 5-BDPU or 6-BDPU
- 0.1 or 1 µM NAA + 1 or 10 µM 5-BDPU or 6-BDPU

Pine cuttings were exposed to the indicated treatments for the entire rooting experiment (28 days).

Statistical analysis

The differences among the mean root numbers were examined with asymptotic Wilcoxon Mann-Whitney rank sum test ($p<0.05$) using R statistics package (R Development Core Team, 2013). The differences among the percentages of rooted cuttings were examined with the Kolmogorov-Smirnov non-parametric test ($p<0.1$) using SPSS Statistic 17.0.

- Adventitious rooting of *Ceratonia siliqua* and *Arbutus unedo* microcuttings

Plant material and in vitro culture conditions

In vitro stock shoot cultures of *Ceratonia siliqua* L. (carob tree) and *Arbutus unedo* L. (strawberry tree) were kindly provided by Dr. Barbara Ruffoni (CRA-FSO, Sanremo, Italy). Carob tree and strawberry tree microcuttings, deprived of apices, were propagated in Murashige & Skoog (1962) medium (MS salts and vitamins: 2 mg l^{-1} glycine, 100 mg l^{-1} myo-inositol, 0.5 mg l^{-1} nicotinic acid, 0.5 mg l^{-1} pyridoxine HCl, 0.1 mg l^{-1} thiamine HCl). Three percent (w/v) sucrose was used as carbon source and medium was solified with 0.8% (w/v) agar. pH was adjusted to 5.8 with 0.1 M NaOH before autoclaving at 121 °C for 20 minutes. Shoot multiplication was established supplementing the medium culture with 0.88 µM BAP for carob tree and 2.88 µM ZR for strawberry tree, respectively. Carob tree and strawberry tree shoots were cultured under 16-h day length at 26 °C, with light provided by cool-white fluorescent lights at a photon flux of 27 µmol m^{-2} s^{-1}. After 6 weeks culture, the new developed shoots were separated and subcultured for further multiplication or used as explants for experiments of root induction.

Adventitious root induction

For root induction, individual shoots of carob or strawberry tree 1.5-2 cm in length, harvested at the end of the multiplication stage, were cultured in tubes containing 15 ml of MS medium, prepared as described above, supplemented with IBA or combinations of IBA and BDPUs for 3 or 7 days of treatment (for compound concentrations and rooting treatments, see **table 4** below). Medium without plant growth regulators (*hormone-free*, HF) was used as control. Only basal portion of each cutting was plunged in the medium. Tubes were capped with aluminium foil and grown at 26 ± 1 °C in the dark to avoid auxin degradation. After 3 or 7 days of treatment, explants were transferred in fresh *hormone-free* MS medium under 16-h day length at normal light conditions (27 µmol m^{-2} s^{-1}). Rooting was evaluated 4 weeks after induction and was expressed in terms of rooting percentage and mean number of adventitious roots per rooted microcutting. For carob, each treatment consisted of approximately 5-15 microcuttings and the experiment was repeated twice. For strawberry tree, each treatment consisted of approximately 5-15 microcuttings and the experiment was repeated five times.

Table 4 ◘ Rooting conditions for carob microcuttings:

- 3 days of induction:

- HF
- 0.1 µM IBA
- 1 µM IBA
- 10 µM 5-BDPU or 6-BDPU
- 0.1 µM IBA + 10 µM 5-BDPU or 6-BDPU
- 1 µM IBA + 10 µM 5-BDPU or 6-BDPU

- 7 days of induction:

- HF
- 1 µM IBA alone
- 10 µM 5-BDPU or 6-BDPU alone
- 1 µM IBA + 10 µM 5-BDPU or 6-BDPU

◘ Rooting conditions for strawberry tree microcuttings:

- 3 or 7 days of induction:

- HF
- 10 µM IBA
- 10 µM 5-BDPU or 6-BDPU
- 10 µM IBA + 10 µM 5-BDPU or 6-BDPU.

After for 3 or 7 days of exposure to indicated treatments, both carob and strawberry tree microcuttings were transferred and kept in HF medium up to 28th day.

Carob rooted plantlet acclimatisation

For acclimatisation, carob plantlets rooted as a consequence of 1 µM IBA with or without BDPUs treatment, were rinsed with tap water to remove adhering medium and placed in plastic pots containing a wet mixture of peat and vermiculite (3:1, v/v). The potted plantlets were maintained inside a plant growth humid chamber with a light regime of 8 h darkness at 21 °C and 16 h light provided by cool-white fluorescent lights (27 µmol m^{-2} s^{-1}) at 25 °C. During the next week, relative humidity was gradually reduced and finally, in the 4th week, plantlets were grown at normal greenhouse conditions. The number of surviving plants after 4 weeks was recorded.

Statistical analysis

Differences among the mean root numbers, percentages of rooted cuttings of either carob or strawberry tree and percentages of survival following acclimatisation of carob

rooted plantlets were examined with asymptotic Wilcoxon Mann-Whitney rank sum test (*p<0.1*) using R statistics package (R Development Core Team, 2013).

- Histological analysis of rooting in *Pinus*

For histological analysis of adventitious root formation, hypocotyl cuttings of *P. radiata* were exposed to the treatments listed in **table 5** and harvested at the beginning of the rooting experiment (time 0), after 6, 10 or 13 days. Basal 5 mm segments were cut with a razor blade.

Table 5 ◘ Rooting conditions for histological investigation of 21-day-old *Pinus* cuttings:

- distilled water
- 1 or 10 µM IBA
- 10 µM 5-BDPU
- 1 µM IBA + 10 µM 5-BDPU

Pine cuttings were exposed to the indicated treatments for the entire rooting experiment (13 days) and harvested at the beginning of the rooting experiment (time 0), after 6, 10 or 13 days.

- *Fixation*

Segments were pooled for each treatment and fixed in formalin-acetic acid-alcohol (FAA). For 100 ml FAA, 90 ml 70% (v/v) ethanol, 5 ml glacial acetic acid, 5 ml 40% (w/v) formaldehyde solution were mixed. Samples were then stored in the fixative solution for 12 h at 4°C.

- *Dehydratation*

After fixation, dehydratation was accomplished by passing samples through of increasing alcohol concentrations and water was gradually substituted with an organic solvent. Thus, basal segments were dehydrated in a series of tertiary-butyl-alcohol (100 ml each change):

1) 10 ml butanol, 40 ml 95% (v/v) ethanol, 50 ml distilled water
2) 20 ml butanol, 50 ml 95% (v/v) ethanol, 30 ml distilled water
3) 35 ml butanol, 50 ml 95% (v/v) ethanol, 15 ml distilled water
4) 55 ml butanol, 40 ml 95% (v/v) ethanol,
5) 75 ml butanol, 40 ml 95% (v/v) ethanol
6) 100 ml butanol

7) 100 ml butanol

8) 100 ml butanol

From 1 to 5, samples were kept in the dehydrating solutions for about 2 h each. To ensure that all tissue water has been removed, segments were kept in 100% buthanol for three changes of about 12 h the first one and 24 h the second and the third ones (from 6 to 8).

- *Embedding*

After dehydratation, samples were gradually embedded in melted paraffin at 60 °C (three changes of 12 h each). Next, melted paraffin was poured into an embedding mold and samples were then placed into the mold. When paraffin got cold, the mold was removed and blocks contaning samples were obtained. Blocks were stored at 4 °C.

- *Sectioning and safranin and fast green staining*

Using a rotary microtome (Reichtert-Jung 2040) 10-µM-transverse sections were obtained and about 1-1.5 mm of each sample was cut.

Tissue sections were stained with safranin-fast green.

Samples were inspected by microscope (Leica 4000B). Images were taken by a Leica DC 100 digital camera applied to the microscope.

- Histological analysis of rooting in carob tree

For histological analysis of adventitious root formation, microcuttings of *C. siliqua* were harvested at the beginning of the rooting experiment (time 0) or exposed to the treatments listed in **table 6** for 3 days and then transferred to HF medium. Samples were harvested after 3, 6 or 12 days from the beginning the rooting experiment. Basal 5 mm segments were cut with a razor blade and then fixed, dehydrated, embedded, sectioned and stained as described above for *Pinus*.

Table 6 ◘ Tested treatments for histological investigation of *C. siliqua* microcuttings:
- HF
- 1 µM IBA
- 10 µM 5-BDPU
- 1 µM IBA + 10 µM 5-BDPU

Carob tree microcuttings were exposed to the indicated treatments for 3 days. Explants were then transferred and kept in HF medium. Microcuttings were harvested at the beginning of the rooting experiment (time 0), after 3, 6 or 12 days.

- IAA immunolocalisation in *Pinus*

Basal 5 mm segments of hypocotyl cuttings, exposed to the treatments listed in **table 7** and harvested after 24 hours, 6 or 10 days from the beginning of the rooting experiment, were collected.

Table 7 ◘ Tested conditions for auxin immunolocalization in *Pinus* cuttings:
- time 0
- 24 hours: 1 µM IBA, 10 µM 5-BDPU, 1 µM IBA + 10 µM 5-BDPU
- 6 days: 1 µM IBA, 10 µM 5-BDPU, 1 µM IBA + 10 µM 5-BDPU
- 10 days: 1 µM IBA, 10 µM 5-BDPU, 1 µM IBA + 10 µM 5-BDPU

- *Tissue fixation*

To perform tissue fixation, a chemical method was adopted. Firstly, basal 5 mm segments of the hypocotyl cuttings were vacuum-infiltrated at room temperature for few minutes with 4% (w/v) paraformaldehyde in 1X phosphate-buffered saline (PBS) [1X PBS: 137 mM NaCl, 2.7 mM KCl, 10.1 mM Na_2HPO_4, 1.76 mM KH_2PO_4, 0.005% (v/v) DEPC, pH 7.4] and then kept in the fixative solution overnight (o/n) at 4 °C. Afterwards, samples were washed three times with 1X PBS (5 minutes each step) and stored with 0.1% (w/v) paraformaldehyde in 1X PBS at 4 °C. For cryoprocessing, samples were cryoprotected with increasing concentrations of sucrose: 0.1 M (1 h), 1 M (1 h) and 2.3 M at 4 °C o/n. At this point, cryoprotected pieces were placed on a metal stub and embedded in Jung Tissue Freezing Medium™ (Leica Microsystems Nussloch GMBH, Heildelberg, Germany) at -25 °C. Pieces were embedded standing with the marked upper part downwards and the basal

part upwards. Thus, the lowest part of 5 mm basal hypocotyl cuttings was cut. 30-40-µm transverse sections were obtained and collected on 3-aminopropyl-triethoxisilan multiwell glass slides. A cryostat (Leica cm 1850 uv) set on -25 °C was used for cryosectioning. Then, glass slides were stored at -20 °C.

- *Permeation*

From permeation step to final mounting slides, each tissue section contained in the well of the multiwell glass slides was covered with a single drop of solvent and expelled by gentle air pressure delivered throught the outer needle of a vacuum pump. The expelled liquid is replaced immediately by a single drop of fresh solvent. Cryostat sections were dewaxed, dehydrated and hydrated with a 30%, 50%, 70%, 100%, 70%, 50%, 30% (v/v) methanol series in 1X PBS at room temperature (5 minutes each step). Cell walls were digested with 2% (w/v) cellulase in 1X PBS for 1 h at room temperature in a humid chamber and, subsequently, samples were rinsed three times with 1X PBS (5 minutes each step).

- *Primary antibody incubation*

During this step, the endogenous IAA was detected by an anti-IAA antibody, and, then, this primary antibody itself was recognized by a second antibody to which is attached a visual marker. To block unspecific binding sities, sections were pre-incubated in 5% (w/v) bovine serum albumin (BSA) in 1X PBS for 10 minutes at room temperature. For immunolabelling, sections were incubated o/n at 4 °C in a humid chamber with mouse monoclonal anti-indole-3-acetic acid antibody (Sigma, St. Louis, MO, USA) diluited 1:100 with 1% (w/v) BSA in 1X PBS.

- *Secondary antibody incubation*

Sections were rinsed five times with 1% (w/v) BSA in 1X PBS (5 minutes each step). To label the IAA, the red-fluorescent Alexa conjugate (568 rabbit anti-mouse IgG, H+L; CSIC, Madrid, Spain) was used as secondary antibody diluited 1:25 with 1X PBS and applied for 45 minutes at room temperature. To prevent fading of the dye this step was performed in darkness. Afterwards, sections were washed five times with 1X PBS and DNA was stained at room temperature with 4',6-diamino-2-phenylindole (DAPI) in Triton X-100 (5 minutes each step). Next, sections were rinsed three times with 1X PBS, 5 minutes each. After immunolabelling, a coverslip containing a generous bubble-free drop of Mowiol® 4-88 (Sigma, St. Louis, MO, USA) was placed on each multiwell glass slide. Nail-varnish was

used to seal the cover slip on the slide. Finally, slides were stored at -20 °C. Negative controls were performed by omitting the first antibody. Therefore, control sections were incubated by following the same protocol as described above, but with 1% (w/v) BSA in 1X PBS, without primary IAA antibodies.

- *Signal detection*

Fluorescent samples were inspected by confocal scanning microscope (Leica sp5). Images were taken by a digital camera applied to the microscope and analyzed with software Leica-Microsystems LAS AF.

- Histochemical localization of GUS activity in *Arabidopsis DR5::GUS* transgenic plants

Plant material and growth conditions

Arabidopsis thaliana DR5::GUS (Ulmasov et al., 1997) transgenic plants in Col-0 background were used. *DR5::GUS* seeds were kindly provided by Dr. Sabrina Sabatini (Università La Sapienza, Rome, Italy). Experiments were performed with plants homozygous for the reporter gene. Seeds were surface sterilised as described above. Sterile seeds were germinated in Petri dishes on ¼ strength MS medium (MS salts) with 0.8% (w/v) agar, 1% (w/v) sucrose, pH 5.8 supplemented with 50 µg ml^{-1} kanamycin. Seedlings were grown at 26 ± 1 °C under a 16 h light regime (27 µmol m^{-2} s^{-1}) for 4 days. Four day-after-germination *Arabidopsis* seedlings were cultivated for 5 h at 26 ± 1 °C with a light intensity of 27 µmol m^{-2} s^{-1} in liquid media (½ MS salts and vitamins, 15 g l^{-1} sucrose, pH 5.8) in multiwell dishes in the presence of the conditions listed below in **table 8**.

Table 8 ◘ Conditions assayed to induce GUS activity in *DR5::GUS Arabidopsis* seedlings:
- DMSO
- 10, 100 nM, 1, 10 or 100 µM 5-BDPU
- 10 or 100 nM IBA or NAA
- 10 or 100 nM IBA + 10 nM or 100 µM 5-BDPU
- 10 or 100 nM NAA + 10 nM or 100 µM 5-BDPU

Arabidopsis seedlings were exposed to the indicated treatments for 5 h.

Histochemical analysis of GUS activity

After 5 h of treatment in liquid media, plantlets were transferred in tubes containing 500 µl of GUS substrate solution [GUS substrate solution: 0.5 mg ml^{-1} 5-bromo-4-chloro-3-indolyl-β-D-glucuronide (Inalco, Milan, Italy) in 100 mM sodium phosphate buffer at pH 7.0, 10 mM EDTA, 1 mM $K3/K4(FeCN)_6$, 0.1% (v/v) Triton X-100, 1% N,N-dimethylformamide]. Samples were stained for 5 h at 37 °C after being placed under a vacuum for 15 minutes. After 5 h staining, samples were washed three times with distilled water to remove excess substrate and destained with absolute ethanol:acetic acid (6:1, v/v) solution overnight at 4 °C. Next, samples were washed twice in 96% ethanol, and stored in 70% (v/v) ethanol. 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.

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

Basal 10 mm segments of hypocotyl cuttings, exposed to the treatments listed in **table 9** and harvested after 24 hours from the beginning of the rooting experiment, were cut with a scalpel blade. Segments from 30 cuttings were pooled for each treatment and immediately frozen in liquid nitrogen and stored at –70° C until used for RNA isolation.

Table 9 ◘ Tested treatments for *qRT-PCR* of *PrSCL1* and *PrSHR* genes:
- distilled water
- 1 µM or 10 µM IBA
- 10 µM 5-BDPU or 6-BDPU
- 1 µM IBA + 10 µM 5- or 6-BDPU

Pine cuttings were exposed to the indicated treatments for 24 hours and then harvested. Hypocotyl cuttings sampled at the beginning of the rooting experiment (time 0) were also harvested as additional control.

- *RNA extraction*

Total RNA was extracted with Plant Concert™ RNA reagent (Invitrogen, Carlsbad, CA, USA) following the manufacturer's instructions. Firstly, frozen segments were ground in liquid

nitrogen to a powder using mortar and pestle and transferred to cool RNase-free polypropilene tubes. 5 ml cold Plant Concert™ RNA Reagent was then added per 1 g frozen, ground tissue into each tube and mixed by vortexing to resuspende the sample. To complete resuspension, tubes were incubated few minutes at room temperature and then centrifugated at 10000 rpm for 5 minutes at 4°C. Afterwards, each supernatant was passed throught a sterilised muslin and filtrates were collected in clean RNnase-free tubes. Next, 2 ml of 5 M NaCl per 10 ml supernatant was added in each tube and mixed by inverting the tube. Subsequently, samples were centrifugated at 10000 rpm for 30 minutes at 4 °C. The upper, aqueous phase was then transferred to a clean RNase-free tube and its volume was measured. 0.9 volume of isopropyl alcohol was added for each sample, mixed and let stand at room temperature for 10 minutes. After, samples were spun at 10000 rpm for 30 minutes at 4 °C. Supernatant was decanted and the pellet was preserved for subsequent steps. For concentrating and de-salting nucleic acids, 1 ml 70% (v/v) RNase-free ethanol was added to each pellet. Tubes were then centrifugated at 10000 rpm for 5 minutes at 4 °C and supernatant was discarded. The residual ethanol was removed by repeating this step. Finally, pellet was dried at room temperature. RNase-free water was added to the RNA pellet and RNA was resuspended by pipetting the liquid up and down over the pellet. RNA resuspended in water was stored at -20 °C. The RNA concentration was then determined using a NanoDrop ND-1000 spectrophotometer (Nucliber, Madrid, Spain), and RNA integrity was checked in a 1% (w/v) agarose gel.

- *DNase treatment*

To increase the sensitivity of the subsequent cDNA synthesis and PCR reactions, contaminating DNA was removed from RNA preparations with the RQ1 RNase-free DNaseI (Promega, Madison, WI, USA). Four micrograms of the total RNA was treated with 4 units of RQ1 RNase-free DNaseI at 37 °C for 30 minutes. To terminate the DNase reaction, tubes were then incubated at 65 °C for 10 minutes. To purify RNA, the total RNA was transferred into Microcon YM-100 (Millipore Corporation, Billerica, MA, USA) tubes and they were spun at 2000 rpm for 15 minutes at 25 °C. To elute RNA, 100 µl of RNase-free water were added to the center of the membrane Microcon and columns then were centrifugated at 2200 rpm for 15 minutes at 25 °C. Sample reservoir was placed upside down and spun at 4000 rpm per 3 minutes at 24 °C to transfer concentrate to vial. Finally, the RNA concentration was determined spectrophotometrically, and RNA integrity was

checked in a 1% (w/v) agarose gel. Pine RNA was prepared from three biological repetitions.

- *cDNA synthesis*

The total RNA was used to perform the synthesis of complementary DNA (cDNA). First strand cDNA synthesis was performed using SuperScript™ II Reverse Transcriptase (Invitrogen) as described in the protocol provided with the kit with slight modifications. Firstly, tubes containing a mixture of 100 ng μl^{-1} random primers and 500 µg of total RNA were heated at 70 °C for 10 minutes and then cooled on ice for 2 minutes to denaturate and anneal the primers to the RNA template. The contents of the tube were then collected by brief centrifugation. Afterwards, 5X first-strand buffer, 0.1 M dithiothreitol (DTT), an enzyme stabilization reagent, 2.5 mM $MgCl_2$ and 1 mM dNTP mix were added to each tube. The contents of the tube were mixed by gently vortexing and incubated at 25 °C for 10 minutes and then at 42 °C for 2 minutes. Next, 200 units of SuperScript™ II RT were added and mixed by pipetting gently up and down. Tubes were incubated at 42 °C for 50 minutes. The activity of the enzyme was terminated by incubating the reaction at 70 °C for 15 min. Samples without SSC II were used as controls.

- *qRT-PCR*

The cDNA was used as a template for amplification in PCR. Newly synthesised PCR products in real-time PCR were detected using SYBR Green I fluorescence dye that binds specifically to the minor groove double-stranded DNA. *PrSCL1* (accession number DQ683567) and *PrSHR* (accession number EU044786) specific primers were designed based on the pine RACE sequences obtained in the lab of prof. Carmen Díaz-Sala (University of Alcalá, Madrid, Spain) as described by Sánchez *et al.* (2007) and Solé *et al.* (2008), respectively (for primer sequences see the **table 10** below). As housekeeping gene control, 18S rRNA gene (*Ri18S*) was used. 18S rRNA primers were designed based on the sequence of *Pinus wallichiana* 18S rRNA (gi: 403026), and confirmed in pine. cDNA samples were run in an optical 96-well plate in a real time PCR machine (ABI-PRISM 7000 Sequence Detection System, Applied Biosystems) using 400 nM target specific primers (*PrSCL, PrSHR, 18S rRNA*), distilled water, and SYBR® Green Master Mix reagent (Applied Biosystems). Reactions were incubated with the standard thermal profile: 50 °C for 2 min, 95 °C for 10 min, 40 cycles of 95 °C for 15 s and 60 °C for 1 min. Three technical replicates from each treatment were subjected to each PCR run. To compare data from different

PCR runs or cDNA samples, the mean of the crossing point (CP) values, defined as the point at which the fluorescence rises appreciably above the background fluorescence, of the three technical replicates was normalized to the mean *CP* value of *Ri18S*. Thus, *PrSCL1* or *PrSHR* gene expression was normalized to that of *Ri18S* by subtracting the *C*T value of *Ri18S* from the *C*T value of the *PrSCL1* or *PrSHR* gene. Relative expression results between treatments in real-time PCR were then obtained from 'Delta-delta method' (Pfaffl, 2001). According to this model, real-time amplification efficiencies (E) of target and reference gene of E=2 were presumed. Thus, the relative expression ratio (R) of a target gene is calculated by the formula $R = 2^{-\Delta\Delta CP}$, where ΔΔCP represents the ΔCP of *PrSCL1* or *PrSHR* for each treatment minus ΔCP of *PrSCL1* or *PrSHR* at time 0. Results are expressed as mean values (± standard error) from three biological replicates.

Table 10 ◘ Pairs of primers used to perform *qRT-PCR* reactions:

- 18S rRNA:
Ri18StrFor 5'-GCGAAAGCATTTGCCAAGG-3'
Ri18StrRev 5'-ATTCCTGGTCGGCATCGTTTA-3'

- Pinus radiata SCARECROW-LIKE1 gene:
PrSCL1trFor 5'-TCAATGTCTGGCAAATCGTCC-3'
PrSCL1trRev 5'-GCGCCCAGTCTCTTCAATTCT-3'

- Pinus radiata SHORT-ROOT gene:
PrSHRtrFor1 5'-GAACCAGTGCAAGGAGCATTG-3'
PrSHRtrRev1 5'-AAATCCTGCCTCCTTGAGCCT-3'

Statistical analysis

For the expression results, significantly different means ($p<0.01$) were identified by using Student's *t* test (SPSS Statistic 17.0).

BDPUs and cytokinin signalling pathway

- Heterologous bacterial assay

Growth conditions

E. coli strain KMI001, harbouring vector pINIII-AHK4 expressing the *Arabidopsis* cytokinin receptor CRE1/AHK4, that is capable of signalling the downstream YojN→RcsB→*cps::lacZ* pathway in response to external cytokinins, was used (Suzuki *et al.*, 2001; Yamada *et al.*, 2001). Bacterial strains were kindly provided by dr. T. Mizuno (University of Nagoya, Nagoya, Japan). Bacteria were grown in Luria broth (10 g l^{-1} tryptone, 5 g l^{-1} yeast extract and 10 g l^{-1} NaCl, pH was adjusted to 7.0 with 1 M NaOH before autoclaving at 121 °C for 20 minutes) at 37 °C with ampicillin (50 µg/ml) with extensive shaking. Culture density was controlled by measuring the absorption at 600 nm (OD_{600}). A homogenous bacterial suspension (OD_{600} between 0.04 and 0.4) was aliquoted in multiwell dishes supplemented with cytokinins and BDPUs (for treatments assayed to induce β-galactosidase activity see **table 11**).

Table 11 ◘ Induction of β-galactosidase activity in CRE1/AHK4 expressing *E. coli*:
- DMSO
- 0.1, 1 or 10 µM TDZ, 2iP or BAP
- 0.1, 1 or 10 µM 5-BDPU or 6-BDPU
- 1 µM TDZ, BAP or 2iP + 0.1, 1 or 10 µM 5-BDPU or 6-BDPU

CRE1/AHK4-expressing *E. coli* were incubated for 40 hours with the indicated treatments

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Binding assay

After 40 h incubation at 25 °C in the dark, the β-galactosidase activity was detected as described by Zhang and Bremer (1995) with minor modifications. Twenty µl samples of cultures were mixed into 80 µl of permeabilization solution (0.8 mg/ml hexadecyltrimethylammonium bromide, 0.4 mg/ml sodium deoxycholate, 100 mM Na_2HPO_4, 20 mM KCl, 2 mM $MgSO_4$, 5.4 µl/ml β-mercaptoethanol). This mixture was kept at 30 °C for 1.30 h, and then 600 µl of 30 °C prewarmed substrate solution (60 mM Na_2HPO_4, 40 mM NaH_2PO_4, 1 mg/ml *o*-nitrophenyl-β-D-galactopyranoside, 2.7 µl/ml β-

mercaptoethanol) were added to initiate the reaction. After 2 h at 30 °C, reactions were stopped by the addition of 700 µl of 1 M Na_2CO_3 and the absorption at 420 nm was recorded for each sample. Enzyme activities were expressed as Miller units (Miller, 1972) by the formula 1000 x [OD_{420}/(OD_{600} x 20 µl x 120 min)]. Experiments were carried out in triplicate and repeated twice.

Statistical analysis

All data were first submitted to logarithmic or Box-Cox power transformation (Box and Cox, 1964). Statistical analysis was performed by analysis of variance (ANOVA) and the significantly different values (*p<0.01*) were identified by using the Tukey's HSD test. Before the ANOVA the hypothesis of homoscedasticity was tested using the Fligner-Killeen test. The statistical analyses were performed by using R statistics package (R Development Core Team, 2013).

- Histochemical localization of GUS activity in *Arabidopsis* P_{ARR5}*:GUS* transgenic plants

Plant material and growth conditions

Transgenic *Arabidopsis* plants, harbouring the *GUS* reporter gene fused to 1.6 kb of the *ARR5* (P_{ARR5}*:GUS*) gene promoter (D'Agostino *et al.*, 2000), were used. P_{ARR5}*:GUS* seeds were obtained from the Nottingham *Arabidopsis* Stock Centre (NASC). Experiments were performed with plants homozygous for the reporter gene. Seeds were surface sterilised, germinated and grown as described above. Four-day-after-germination *Arabidopsis* seedlings were cultivated for 5 h at 26 ± 1 °C with a light intensity of 27 µmol m^{-2} s^{-1} in liquid media (½ MS salts and vitamins, 1.5% (w/v) sucrose, pH 5.8) in multiwell dishes in the presence of the conditions listed in **table 12**. The histochemical analysis of GUS activity was performed as described above for *Arabidopsis DR5::GUS* transgenic plants.

Table 12 ◘ Conditions assayed to induce GUS activity in P_{ARR5}*:GUS Arabidopsis* seedlings:

- DMSO
- 1, 10, 100 nM, 1, 10 or 100 µM 5-BDPU
- 1, 10 or 100 nM 2iP or TDZ
- 1 or10 nM 2iP + 1, 10, 100 nM, 1, 10 or 100 µM 5-BDPU
- 1 or10 nM TDZ + 1, 10, 100 nM 5-BDPU

Arabidopsis seedlings were exposed to the indicated treatments for 5 h.

Somatic embryogenesis

- Somatic embryogenesis of *Medicago truncatula*

Plant material and growth conditions

Medicago truncatula line R108 plants were used. Seeds were kindly provided by Dr. Pascal Ratet (Institut des Sciences du Vegetal, CNRS, Gif-sur-Yvette, France). Seeds were gently scarified with soft glass paper. They were then surface sterilized by soaking in 6% (v/v) commercial bleach (equivalent of 0.3% NaOCl) for 10 minutes followed by four time rinses with sterile distilled water. Sterile seeds were germinated by 2 days incubation on moist filter paper in sterile Petri dishes in darkness at 4 °C and then Petri dishes were transferred under 16 h light regime provided by cool-white fluorescent lights (27 µmol m^{-2} s^{-1}) in the growth chamber at 26 °C. The germinated seeds were transferred onto MS medium (MS salts and vitamins: 2 mg l^{-1} glycine, 100 mg l^{-1} myo-inositol, 0.5 mg l^{-1} nicotinic acid, 0.5 mg l^{-1} pyridoxine HCl, 0.1 mg l^{-1} thiamine HCl). Three percent (w/v) sucrose was used as carbon source and medium was solified with 0.8% (w/v) agar. pH was adjusted to 5.8 with 0.1 M NaOH before autoclaving at 121 °C for 20 minutes. After 4 weeks of *in vitro* culture, two-node explants were micropropagated onto MS medium (MS salts and vitamins), 3% (w/v) sucrose, 0.8% (w/v) agar under 16 h light regime (27 µmol m^{-2} s^{-1}) in the growth chamber at 26 °C for further 4 weeks *, while* leaf explants were used for the initiation of somatic embryogenesis.

Somatic embryogenesis induction

Well-expanded leaves from 4-week-old *in vitro*-grown plants were wounded 3 times by scalpel blade across main vein and placed adaxial side up in Petri dishes containing MS medium (MS salts and vitamins), 3% (w/v) sucrose, 0.7% (w/v) agar supplemented with 1 g l^{-1} casein hydrolysate. pH was adjusted to 5.8 with 0.1 M NaOH before autoclaving at 121 °C for 20 minutes. The medium was then supplemented with the compounds listed below (see **table 13** for compound concentrations and embryo induction conditions). Each Petri dish contains 6 leaflets. Two Petri dishes were used for each condition. The experiments were repeated twice. Explants were subcultured every two weeks on fresh embryo induction medium and maintained under 16 h light regime (27 µmol m^{-2} s^{-1}) in the growth chamber at 26 °C till 40 days from the beginning of the experiment.

Somatic embryo conversion and rooting

As embryos appeared, they were transferred to embryo conversion and rooting (ECR) medium (MS salts and vitamins), 250 mg l^{-1}casein hydrolysate, 1% (w/v) sucrose and 0.7% (w/v) agar). pH was adjusted to 5.8 with 0.1 M NaOH before autoclaving at 121 °C for 20 minutes. The medium was then supplemented with 0.537 µM NAA. Developing plantlets were subcultured every 14 days on fresh ECR medium and maintained under 16 h light regime (27 µmol m^{-2} s^{-1}) in the growth chamber at 26 °C for a period of 40 days.

Table 13 ◘ Embryo induction treatments used for plant regeneration of *Medicago truncatula* line R108:

- 4.52 µM 2,4-D
- 0.88 µM BAP
- 4.52 µM 2,4-D + 0.88 µM BAP
- 4.52 µM 2,4-D + 0.88 µM BAP + 1, 10, 20 or 40 µM 5-BDPU or 6-BDPU
- 4.52 µM 2,4-D + 1, 10, 20 or 40 µM 5-BDPU or 6-BDPU
- 0.88 µM BAP + 1, 10, 20 or 40 µM 5-BDPU or 6-BDPU

Statistical analysis

Differences among the mean numbers of embryos per explant, percentages of reactive explants and percentages of conversion of embryos to plantlets were examined with

asymptotic Wilcoxon Mann-Whitney rank sum test (*$p \leq 0.05$*) using R statistics package (R Development Core Team, 2013).

Shoot regeneration

- Regeneration test in *Lycopersicon esculentum*

Plant material and growth conditions

Seeds of the commercial variety (Alice) of *Lycopersicon esculentum* were surface sterilized by soaking in 70% (v/v) ethanol for 30 seconds and 20% (v/v) commercial bleach for 20 minutes (equivalent to 1% NaOCl) and then washed 3 times with sterilized water. Seeds were sown in glass culture pots containing ¼ MS (salts) supplemented with 0.8% (w/v) agar. pH was adjusted to 5.8 with 0.1 M NaOH before autoclaving. Pots were placed in a growth chamber at 26 ± 1 °C at light intensity of 27 µmol m^{-2} s^{-1} under 16-h day length.

Shoot regeneration induction

Cotyledons from one-week old seedlings were cut by scalpel blade. Two explants from each cotyledon were obtained. Twelve cotyledons explants were plated in each Petri dish containing MS medium (MS salts and vitamins, 3% (w/v) sucrose, 0.8% (w/v) agar) and the compounds listed below (for compound concentrations and regeneration conditions see **table 14** below). Petri dishes were then incubated at 26 ± 1 °C at light intensity of 27 µmol m^{-2} s^{-1} under 16-h day length. After two-weeks of incubation, cotyledon explants were transferred in *hormone-free* medium and the percentage of the explants forming shoots was checked two weeks later. The experiments were done in triplicate and repeated three times.

Table 14 ◘ Shoot regeneration treatments of tomato cotyledon explants:

- 1.4 µM ZR + 20 µM BOAA
- 1, 5 or 10 µM 2iP, BAP or TDZ
- 1, 5 or 10 µM 2iP + 0.1, 1 or 10 µM 5-BDPU
- 1, 5 or 10 µM BAP + 0.1, 1 or 10 µM 5-BDPU
- 1, 5 or 10 µM TDZ + 0.1, 1 or 10 µM 5-BDPU

Tomato cotyledon explants were exposed to these treatments for two weeks and then transferred to HF medium for two weeks.

Statistical analysis

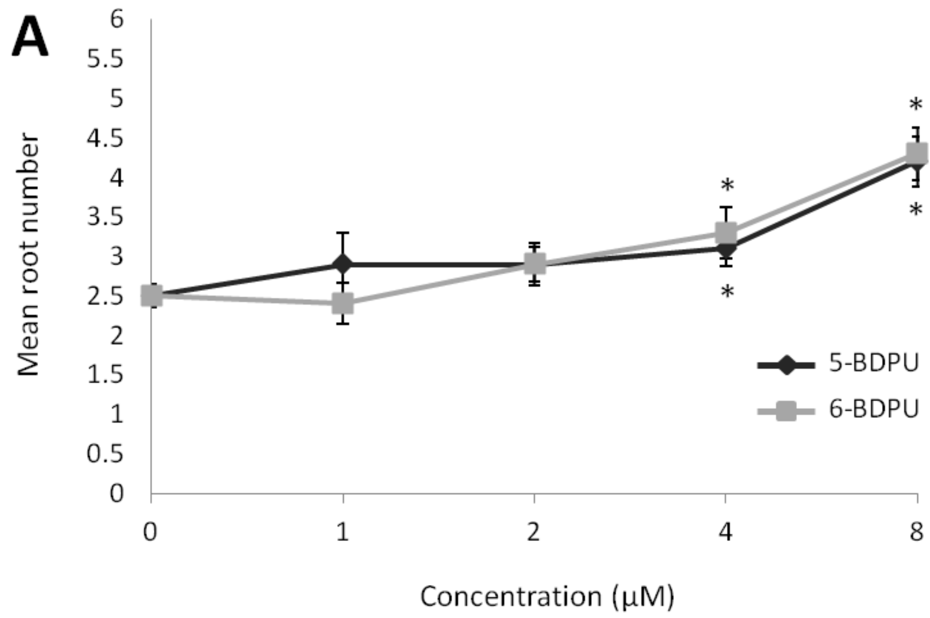
Differences among the percentages of cotyledon explants forming shoots were examined with asymptotic Wilcoxon Mann-Whitney rank sum test ($p<0.05$) using R statistics package (R Development Core Team, 2013).

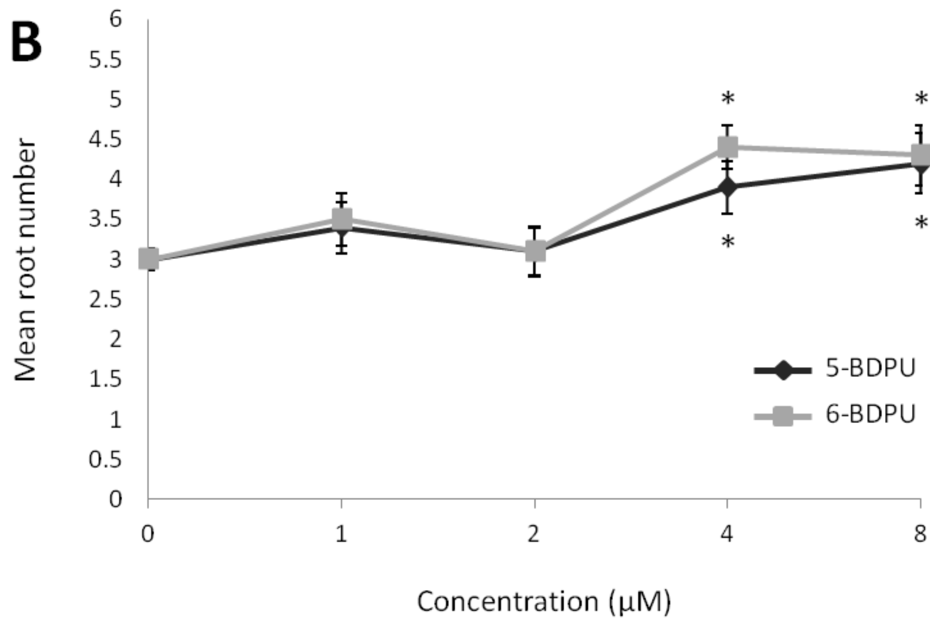
RESULTS

Adventitious rooting

In order to determine if the adventitious rooting adjuvant activity exerted by 5-BDPU and 6-BDPU could depend on the type/chemical structure of the exogenous auxins inducing adventitious roots, we verified the effect of BDPUs in combination with IBA and NAA. Both types of auxin share a planar ring system. However, while IBA is a natural occurring auxin carrying an indole ring, NAA is a synthetic naphthalene derivative. We tested mixtures of these auxins and BDPUs in herbaceous and woody plants using different experimental systems, such as etiolated seedlings of the model plant *Arabidopsis thaliana* and *Pinus radiata* hypocotyl cuttings. Moreover, we examined the effectiveness of the mixtures made by BDPUs plus NAA in apple stem slice system. This experimental system represents an excellent tool to study adventitious rooting since it avoids any interference from other parts of the shoot and therefore the rooting response is only the result of the type and concentration of the compounds supplied in the medium. With the aim of studying the spectrum of action of BDPUs, we analysed the effect of BDPUs in combination with IBA on the adventitious rooting capacity of difficult to root carob and strawberry tree microcuttings. Trying to understand the mode of action of these compounds during the adventitious rooting process, we analysed their interaction with auxin signalling pathway in *Pinus*. The cooperation between auxins and BDPUs was also investigated by carrying out a histological time course of the cellular events giving rise to the formation of adventitious roots in both *Pinus* and carob. Finally, we evaluated the endogenous auxin distribution in *Pinus* and *Arabidopsis* in response to combinations of auxins and BDPUs. Since we assumed a similar rooting adjuvant activity for either 5-BDPU or 6-BDPU, some experiments have been performed using 5-BDPU only.

Effect of BDPUs on adventitious rooting of *Arabidopsis* seedlings





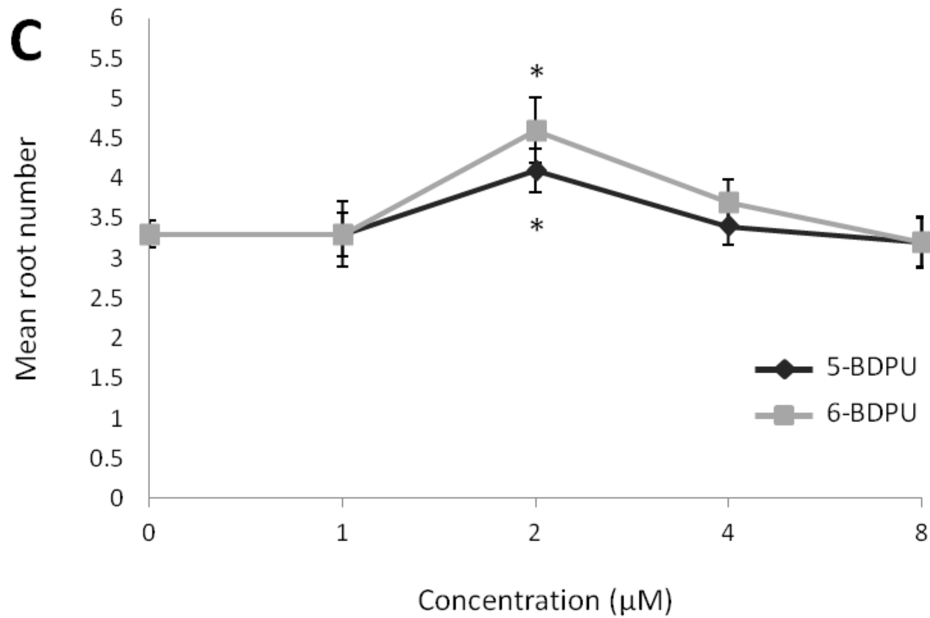


Fig. 9

Adventitious rooting of etiolated *Arabidopsis* seedlings. (A) Mean root number obtained in the presence of 1, 2, 4, 8 μM 5-BDPU or 6-BDPU supplemented alone in the culture medium. Control was performed with DMSO alone. (B) Mean root number obtained in the presence of 1, 2, 4, 8 μM 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 obtained in the presence of 1, 2, 4, 8 μM 5-BDPU or 6-BDPU in the simultaneous presence of 0.1 μM NAA. Control was performed with 0.1 μM NAA alone. The number of root was counted after 7 d. Asterisks indicated significant difference between treatments and control at $p<0.05$ level by Student's *t*-test. Error bars show SE (n=6).

In order to verify the effect of BDPUs on adventitious rooting in *Arabidopsis*, etiolated seedlings were cultured for 7 days in the presence of increasing concentration of 5-BDPU or 6-BDPU alone or in combination with 0.1 μM IBA or NAA. 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) (**Fig. 9A**). 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) (**Fig. 9B**). 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 versus 3.3) (**Fig. 9C**).

Effect of BDPUs on adventitious rooting of *Malus pumila* stem slices

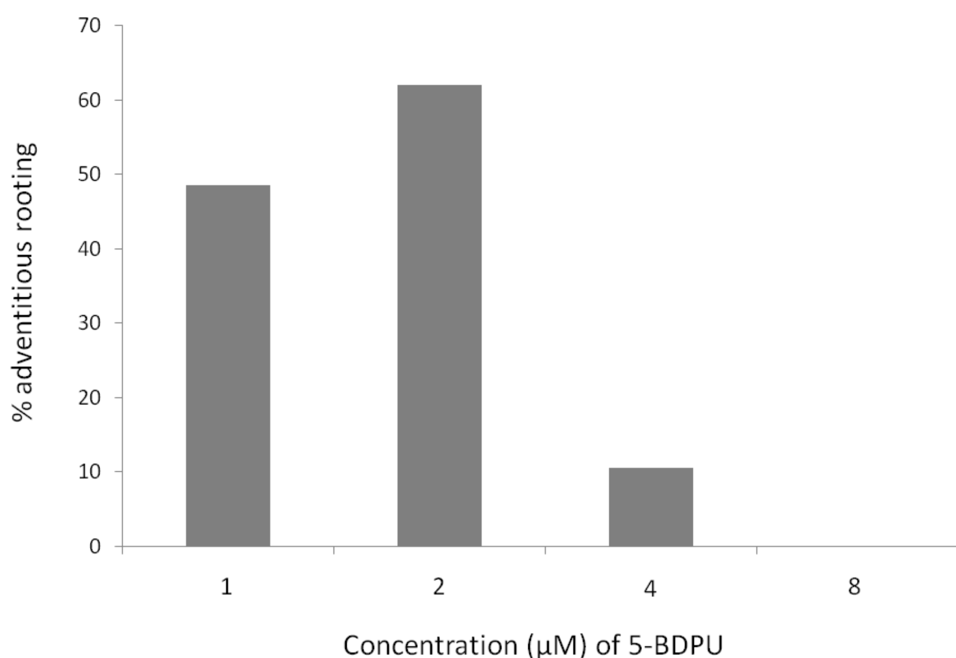


Fig. 10
Effect of different concentrations of 5-BDPU on the percentage of adventitious rooting of apple stem slices. During this treatment, 1 µM NAA was also present in the medium. The results were expressed as a percentage of the control (1 µM NAA alone) by the formula [(T-C/C)*100].

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 µM 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 decreased (10.5% over the control at 4 µM) at higher BDPU concentrations (**Fig. 10**). An emerging presence of callus and no roots was observed in each slice in the presence of the highest 5-BDPU concentration (8 µM).

Effect of BDPUs on adventitious rooting of *Pinus* cuttings

Table 15 – Percentage of hypocotyl cuttings of 21-day-old *Pinus radiata* seedlings with adventitious roots in response to 0.1, 1 or 10 µM IBA alone or in combination with 0.1, 1 or 10 µM 5-BDPU or 6-BDPU (A), and in response to 0.1 or 1 µM NAA alone or in combination with 1 or 10 µM 5-BDPU or 6-BDPU (B). Rooting experiments were conducted by placing five cuttings together in glass vials containing tested solutions for 4 weeks. One vial was considered as one observation. Asterisks indicated significant differences between control (auxins alone) and treatments (auxins plus BDPUs) (*p<0.1*, Kolmogorov-Smirnov test)

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

B	**NAA:**	
	0.1 µM	**1 µM**
	55.0	100.0
5-BDPU:		
1 µM	54.6	100.0
10 µM	80.0 *	100.0
6-BDPU:		
1 µM	40.0	100.0
10 µM	60.0	90.0

To assess the effect of different concentrations of 5-BDPU or 6-BDPU on the adventitious root formation of pine cuttings, rooting experiments were conducted by exposing hypocotyl cuttings from 21-day-old *Pinus radiata* seedlings to combinations of BDPUs and 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). The mean root number did not differ in the presence of combinations of either IBA or NAA and BDPUs if compared to the mean root number recorded in the presence of IBA or NAA alone, at any of the tested concentrations of either auxins or BDPUs (data not shown). Data presented in **Table 15** show the effect of 5-BDPU or 6-BDPU plus IBA (**Table 15A**) or NAA (**Table 15B**) on the percentage of rooted hypocotyls cuttings. The combinations of 0.1 µM IBA plus 0.1, 1 or 10 µM BDPUs were ineffective and the percentages of rooted cuttings were not significantly different from that of the same IBA concentration alone (**Table 15A**). No callus formation was observed at the base of the cuttings. In the presence of 10 µM IBA plus 0.1, 1 or 10 µM BDPUs 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 percentage of rooted cuttings was only enhanced in the presence of the mixtures made by 1 µM IBA and 10 µM BDPUs, since the percentages recorded with these treatments were significantly higher than that obtained in the presence of 1 µM IBA alone. No callus formation was observed at the base of the cuttings. The mixtures made by 0.1 µM NAA plus BDPUs significantly increased the percentage of rooted cuttings only when 10 µM 5-BDPU was used. The combinations of 1 µM NAA plus 0.1, 1 or 10 µM BDPUs did not increase the percentages of rooted cuttings from that of the same NAA concentration alone (**Table 15B**). The mixtures made by 0.1 µM NAA and 6-BDPU were ineffective, at all the tested 6-BDPU concentrations. No callus formation was observed at the adventitious root emergence sites in the mixtures made by 0.1 or 1 µM NAA plus BDPUs.

Effect of BDPUs on adventitious rooting of *Ceratonia siliqua* microcuttings

Table 16 – Rooting percentage (% R) and number of adventitious roots per rooted microcutting (mean ± SE) from carob tree plantlets exposed for 3 days to 1 µM IBA with or without 10 µM 5-BDPU or 6-BDPU. For mean root number, the number of observations was 4. Asterisk indicate significant difference between treatments and control at *p<0.1* by WMW test; n = 2.

	1 µM IBA	
	% R	Mean root number
	47.6	5.3 ± 1.4
+ 10 µM 5-BDPU	73.3 *	5.2 ± 1.2
+ 10 µM 6-BDPU	61.1	4.9 ± 1.5

Table 17 – Rooting percentage (% R) and number of adventitious roots per rooted microcutting (mean ± SE) from carob tree plantlets exposed for 7 days to 0.1 or 1 µM IBA with or without 10 µM 5-BDPU or 6-BDPU. No significant differences among treatments and controls were found (*p<0.1*, WMW test); n = 2.

	IBA:			
	0.1 µM		**1 µM**	
	% R	Mean root number	% R	Mean root number
	39.5	3.8 ± 0.6	37.9	2.4 ± 0.4
+ 10 µM 5-BDPU	41.2	3.7 ± 1.3	34.3	4.0 ± 2.0
+ 10 µM 6-BDPU	42.9	4.1 ± 0.5	46.3	3.8 ± 2.8

Table 18 – Percentage of survival after 4 weeks acclimatisation of carob tree rooted plantlets from *in vitro* rooting experiments. No significant differences among treatments and controls were found (*p<0.1*, WMW test).

	1 µM IBA	
	3 days of treatment	7 days of treatment
	77.8	92.3
+ 10 µM 5-BDPU	83.3	100.0
+ 10 µM 6-BDPU	100.0	100.0

In order to investigate the action spectrum of the BDPUs, their rooting adjuvant activity has been analysed in difficult-to-root carob microcuttings. *In vitro* root induction of microcuttings was performed by supplementing 5-BDPU or 6-BDPU with IBA for two different incubation times (3 or 7 days). **Table 16** shows percentages of rooted microcuttings and mean root number following 3 days of induction in the presence of 1 µM IBA plus 10 µM BDPUs. The mean root number did not differ following exposure to combinations of IBA and BDPUs if compared to that recorded in the presence of IBA alone. However, the combination of 1 µM IBA plus 10 µM 5-BDPU enhanced the adventitious root formation as the percentage of rooted microcuttings was greater than that of the same IBA concentration. Data presented in **table 17** show that neither 5-BDPU nor 6-BDPU applied with 0.1 or 1 µM IBA for 7 days was effective in inducing any enhancement of adventitious rooting. As shown in **table 18**, most of the cuttings rooted for 3 or 7 days in the presence of IBA plus BDPUs were successfully acclimatised to *ex vitro* conditions.

Effect of BDPUs on adventitious rooting of *Arbutus unedo* microcuttings

Table 19 – Percentage of rooted microcuttings from strawberry tree plantlets exposed for 3 or 7 days to 10 µM IBA with or without 10 µM 5-BDPU or 6-BDPU. No significant differences between control (IBA alone) and treatments (IBA plus BDPUs) were found (*p<0.1*, WMW test).

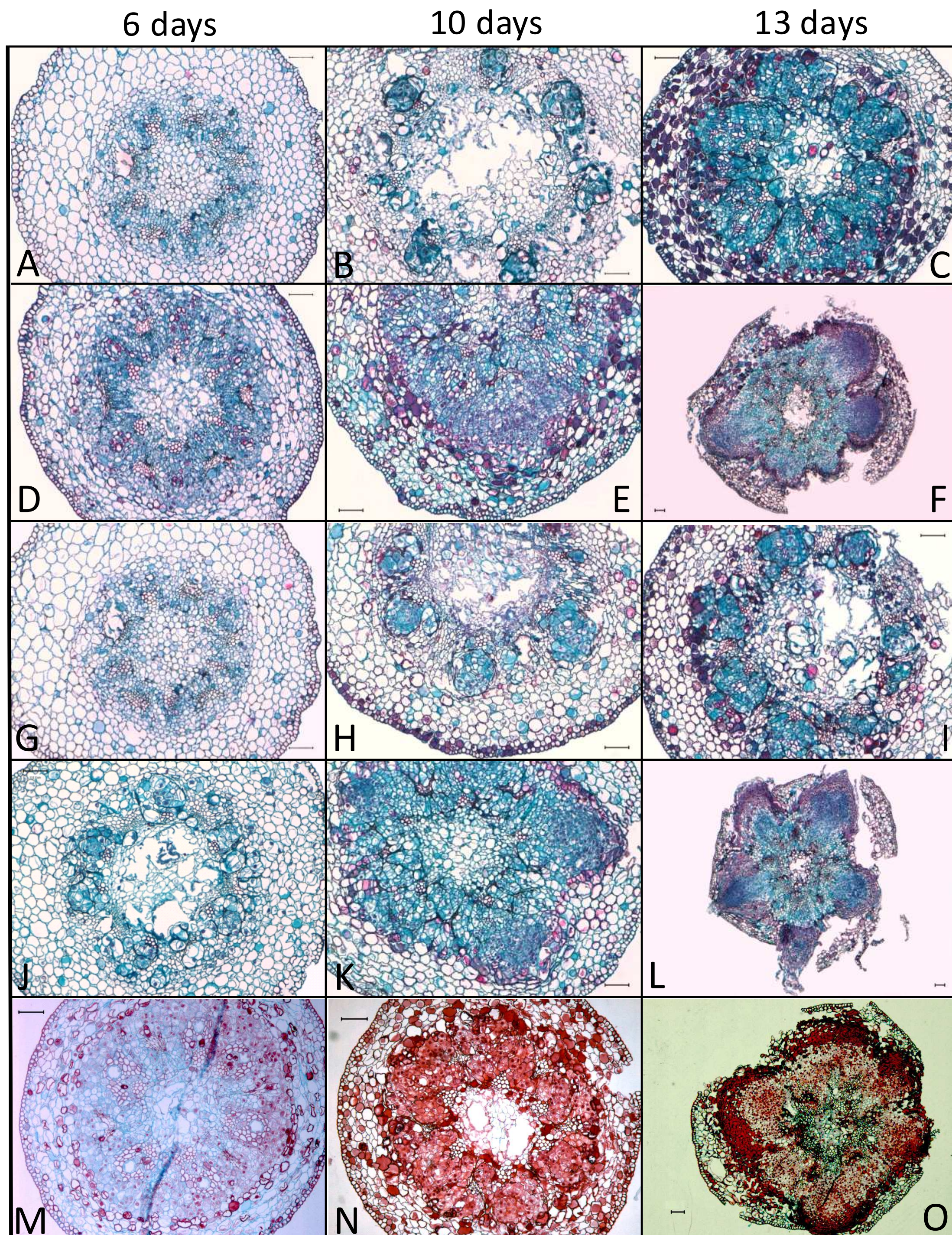
	10 µM IBA	
	3 days of treatment	7 days of treatment
	50.0	64.5
+ 10 µM 5-BDPU	64.4	60.7
+ 10 µM 6-BDPU	51.9	52.1

Table 20 – Mean number of adventitious roots per rooted microcuttings (± SE) from strawberry tree plantlets exposed for 3 or 7 days to 10 µM IBA with or without 10 µM 5-BDPU or 6-BDPU. No significant differences between control and treatments were found (*p<0.1*, WMW test).

	10 µM IBA	
	3 days of treatment	7 days of treatment
	4.0 ± 0.8	3.3 ± 0.7
+ 10 µM 5-BDPU	3.7 ± 0.6	4.3 ± 0.9
+ 10 µM 6-BDPU	4.0 ± 1.0	4.2 ± 0.7

In order to investigate the action spectrum of the BDPUs, their rooting adjuvant activity has been analyzed in difficult-to-root strawberry tree microcuttings. *In vitro* root induction of microcuttings was performed by applying 5-BDPU or 6-BDPU with IBA for two different incubation times (3 or 7 days). As shown in **tables 19 and 20**, none of the tested combinations were able to enhance the formation of adventitious roots at any of the tested incubation times.

Effect of 5-BDPU on cellular events leading to the adventitious root formation of *Pinus*



◄ **Fig. 11**

Cross sections of the base of 21-day-old *Pinus radiata* hypocotyl cuttings cultured in different culture conditions: in the presence of water at day 6 (A), 10 (B) and 13 (C); in the presence of 1 µM IBA at day 6 (D), 10 (E) and 13 (F); in the presence of 10 µM 5-BDPU at day 6 (G), 10 (H) and 13 (I); in the presence of 1 µM IBA plus 10 µM 5-BDPU at day 6 (J), 10 (K) and 13 (L); in the presence of 10 µM IBA at day 6 (M), 10 (N) and 13 (O). Bars indicate 100 µm.

With the aim of understanding how 5-BDPU promoted root formation in *Pinus* cuttings, we performed a histological analysis of the base of cuttings exposed to 1 or 10 µM IBA, 10 µM 5-BDPU and 1 µM IBA plus 10 µM 5-BDPU after 6, 10 and 13 days of treatment. Results are shown in **fig. 11**. Hypocotyls from 21-day-old *P. radiata* seedlings showed a well defined primary structure (see **fig. 11** of the Introduction), consisting in 5-6 poles of primary xylem and phloem marked by a centrifugal resin canal. Cell divisions were observed in the vascular parenchyma after 6 days in all tested conditions (first column of **fig. 11**). Cuttings cultured in water exhibited globular-shaped groups of dividing cells, organized in a sort of 'internal nodules' closely positioned to the resin canals after 10 days of culture (**Fig. 11B**). The nodules subsequently increased their capacity of divisions; however, no root primordia were visible (**Fig. 11C**). At day 6, in 1 µM IBA-treated cuttings, non-organized cell divisions, giving rise to the formation of root primordia (clearly visible at day 13) were observed (**Fig. 11E and F**). Six days after treatment, cuttings exposed to 10 µM 5-BDPU exhibited an internal structure similar to that observed when cuttings were cultured in water. However, the 'internal nodules' located centrifugal to the resin canals were still clearly visible after 13 days of culture (**Fig. 11H and I**). The mixture made by 1 µM IBA plus 10 µM 5-BDPU led to the formation of globular-shaped groups of dividing cells around the resin canals at day 6. Subsequently, these 'internal nodules' developed into root primordia at day 10 and root emergence was observed at day 13 (**Fig. 11J, K and L**). A high rate of non-organized cell divisions was observed after 6 and 10 days of treatment in the presence of 10 µM IBA. Root primordia were visible at day 13 (**Fig 11M, N and O**).

Effect of 5-BDPU on cellular events leading to the adventitious root formation of carob tree

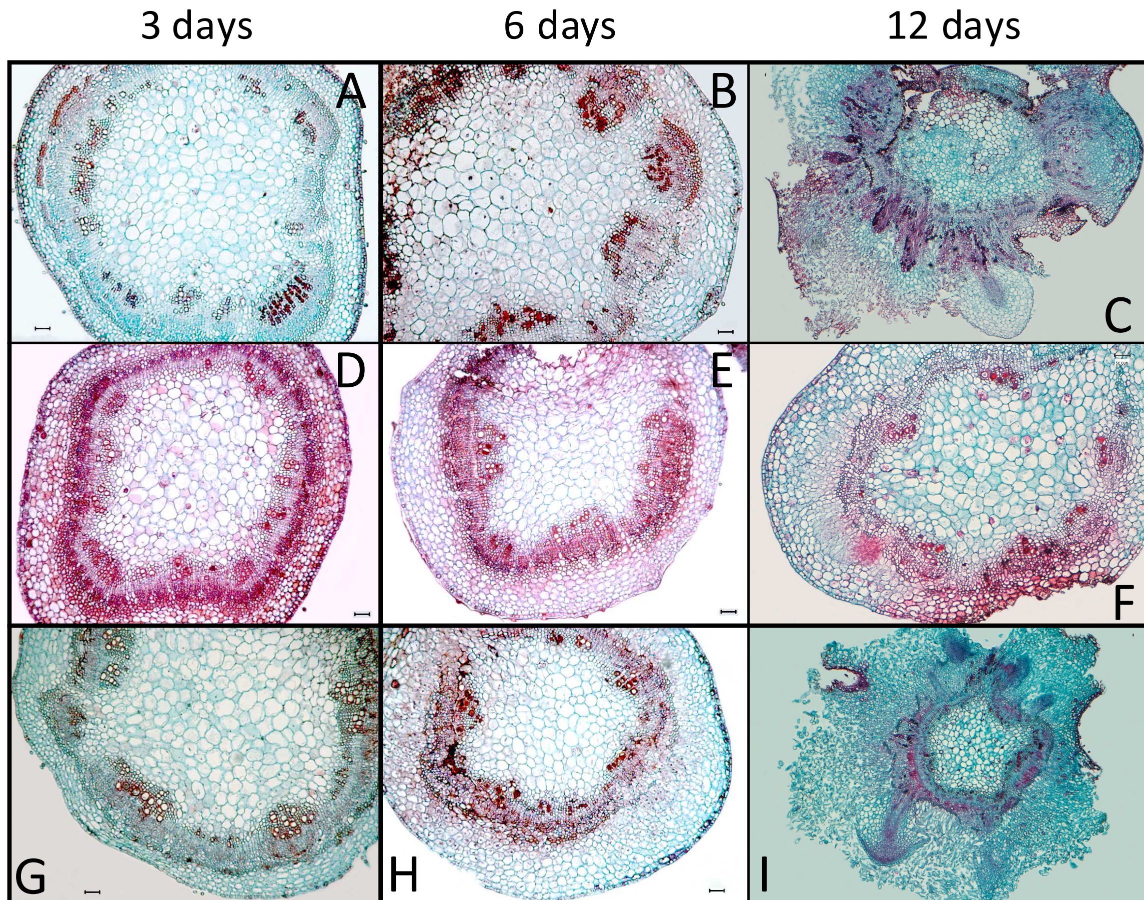


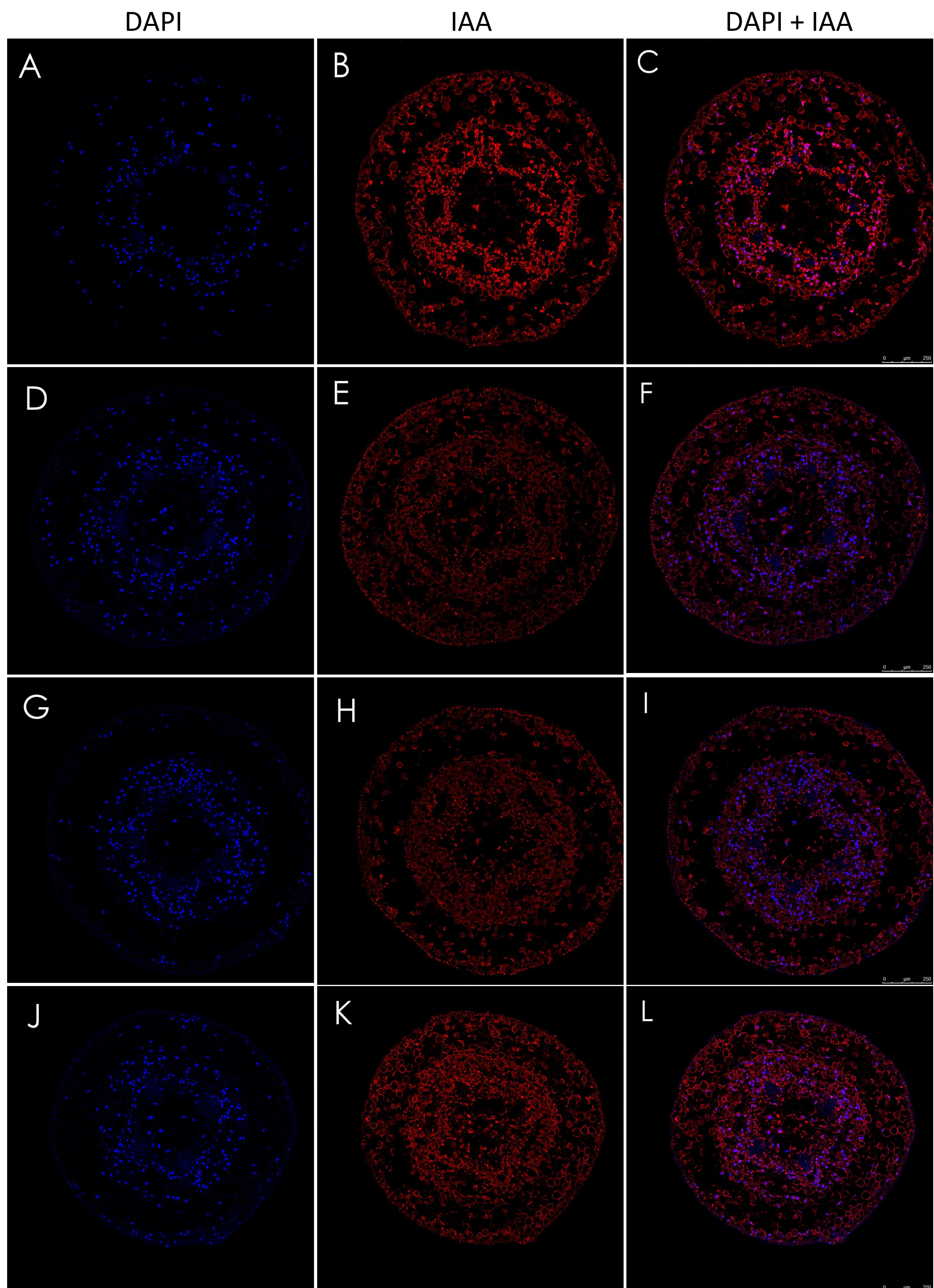
Fig. 12

Cross sections of the base of 6-week-old *Ceratonia siliqua* microcuttings cultured for 3 days in the presence of 1 µM IBA, 10 µM 5-BDPU or 1 µM IBA plus 10 µM 5-BDPU. Base of 1 µM IBA-treated microcutting at day 3 (A), 6 (B) and 12 (C). Base of 10 µM 5-BDPU-treated microcutting at day 3 (D), 6 (E) and 12 (F). Base of 1 µM IBA plus 10 µM 5-BDPU-treated microcutting at day 3 (G), 6 (H) and 12 (I). Bars indicate 50 µm.

In order to anatomically investigate the effect of 5-BDPU on adventitious rooting process in carob tree, we carried out a time course of cellular events giving rise to the formation of adventitious roots in cross sections of the base of IBA and IBA plus 5-BDPU-treated 6-week-old microcuttings (**Fig. 12**). At day 3 and 6 after treatment, no major difference in the anatomical structure of the stem was observed when comparing the microcuttings treated with IBA or 5-BDPU alone to those that were treated with a combination of IBA plus 5-BDPU (**Fig. 12A, B, D, E, G and H**). At day 12, dividing cells

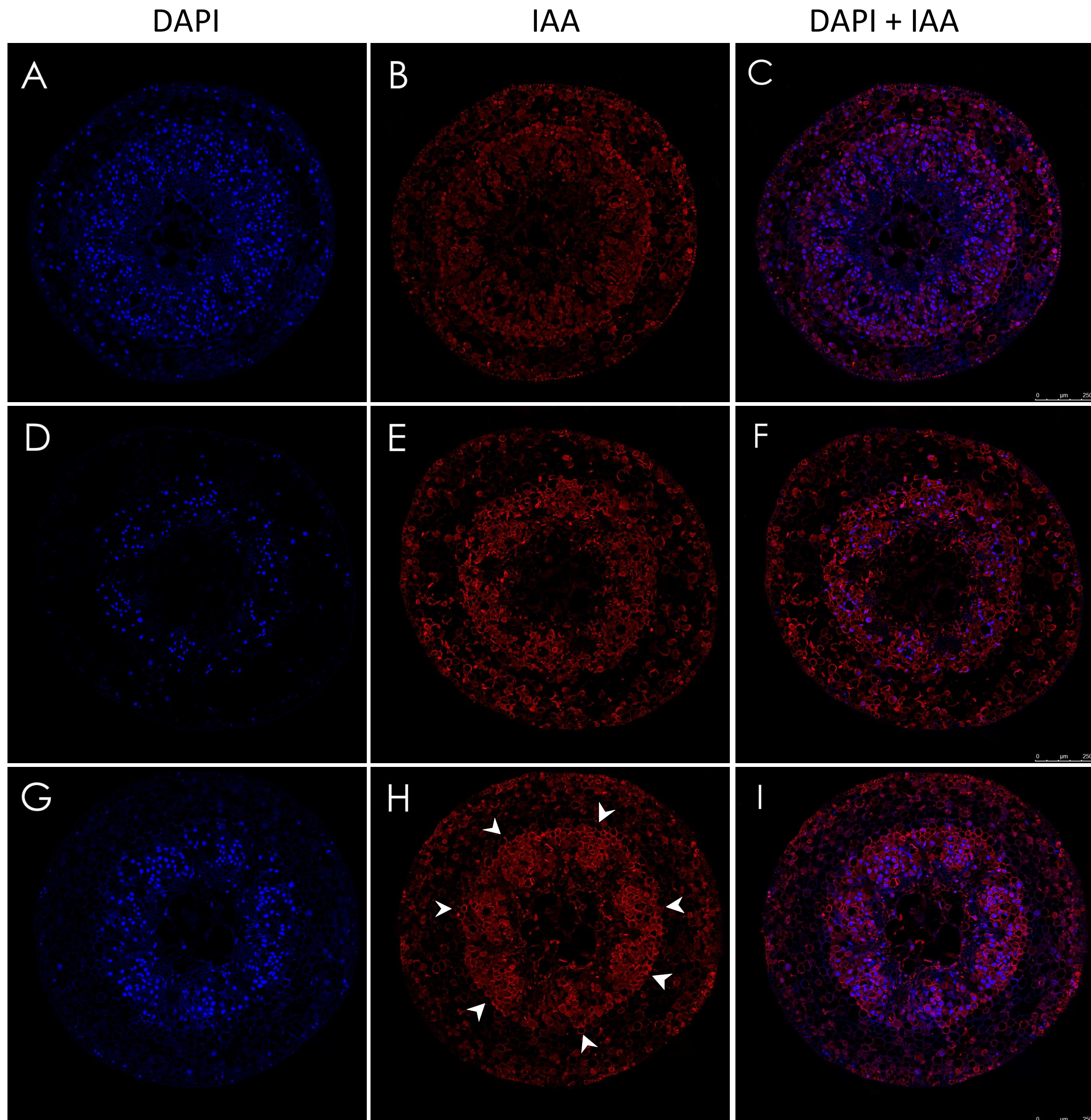
originating from the cambial zone were clearly visible in the presence of 5-BDPU alone, even if this treatment was not able to induce adventitious root formation (**Fig. 12F**). Root primordia emerging from a high rate of non-organized cambial cell divisions producing callus were observed in the presence of IBA alone (**Fig. 12C**), whereas root primordia were initiated directly from cambial derivatives in the simultaneous presence of IBA and 5-BDPU (**Fig. 12I**).

Effect of 5-BDPU on auxin distribution in *Pinus*



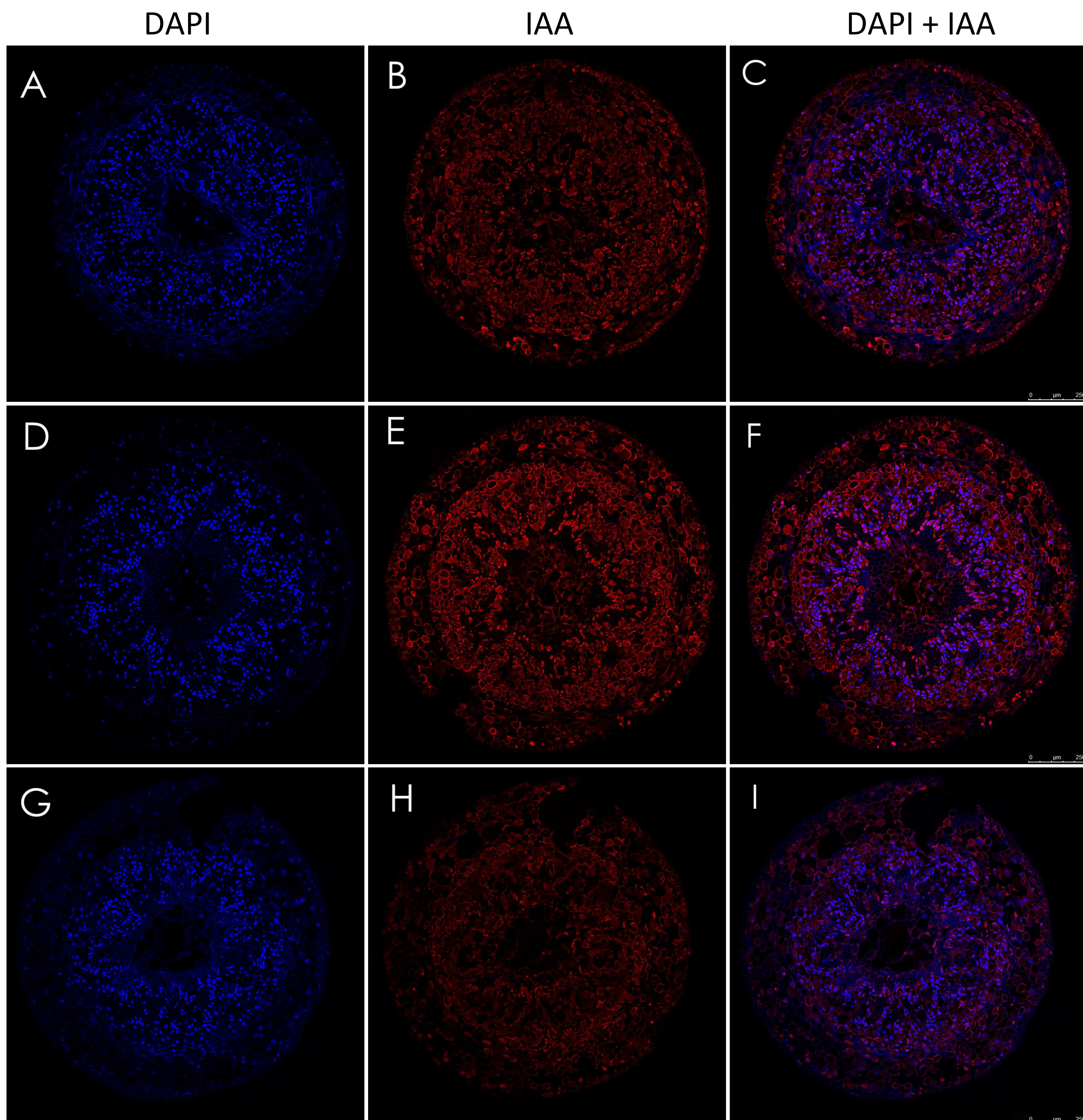
◀ **Fig. 13**

Immunohistochemical localisation of indole-3-acetic acid (IAA) in cross sections of the base of 21-day-old *Pinus* hypocotyl cuttings at <u>1 day</u> of treatment. (A, B and C) Hypocotyl cutting at day 0. (D, E and F) 1 µM IBA-treated cuttings. (G, H and I) 10 µM 5-BDPU-treated cuttings. (J, K and L) 1 µM IBA plus 10 µM 5-BDPU-treated cuttings. In the first column, the DAPI-stained nuclei are depicted in blue; in the second column, IAA signal is depicted in red; in the third column, DAPI and IAA signals are overlapped. Bars in the third column represent 250 µm.



◄ **Fig. 14**

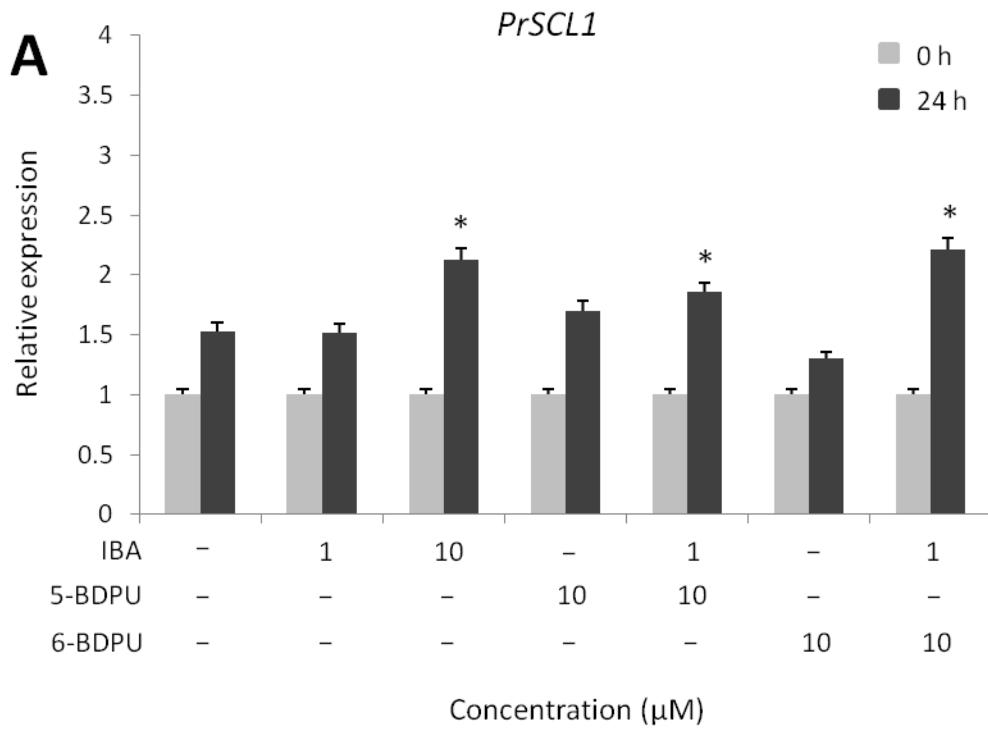
Immunohistochemical localisation of indole-3-acetic acid (IAA) in cross sections of the base of 21-day-old *Pinus* hypocotyl cuttings at 6 days of treatment. (A, B and C) 1 µM IBA-treated cuttings. (D, E and F) 10 µM 5-BDPU-treated cuttings. (G, H and I) 1 µM IBA plus 10 µM 5-BDPU-treated cuttings. In the first column, the DAPI-stained nuclei are depicted in blue; in the second column, IAA signal is depicted in red; in the third column, DAPI and IAA signals are overlapped. Bars in the third column represent 250 µm. *White arrowheads* indicate spots of IAA accumulation around the resin canals.

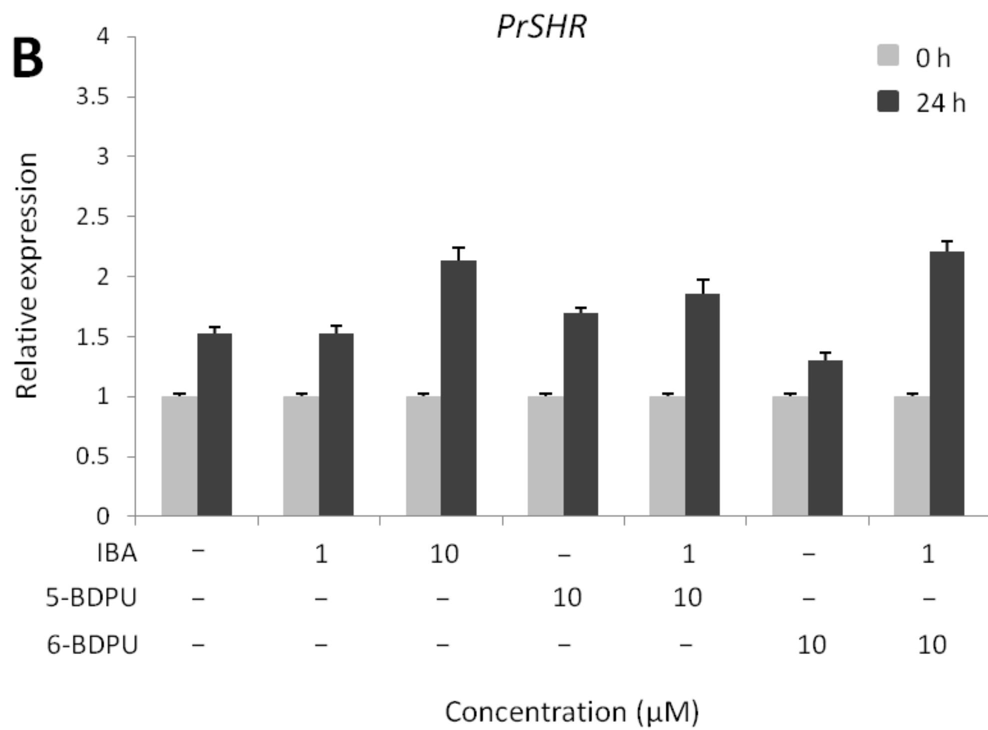


◄ **Fig. 15**
Immunohistochemical localisation of indole-3-acetic acid (IAA) in cross sections of the base of 21-day-old *Pinus* hypocotyl cuttings at 10 days of treatment. (A, B and C) 1 µM IBA-treated cuttings. (D, E and F) 10 µM 5-BDPU-treated cuttings. (G, H and I) 1 µM IBA plus 10 µM 5-BDPU-treated cuttings. In the first column, the DAPI-stained nuclei are depicted in blue; in the second column, IAA signal is depicted in red; in the third column, DAPI and IAA signals are overlapped. Bars in the third column represent 250 µm.

To understand if and how 5-BDPU affects the auxin local pools at the wounded sites of *Pinus* cuttings, we analysed changes in IAA accumulation and distribution during adventitious root induction following exposure to IBA, 5-BDPU or IBA plus 5-BDPU after 1 (**Fig. 13**), 6 (**Fig. 14**) and 10 (**Fig. 15**) days of treatment. After 1 day of treatment, no difference in endogenous IAA distribution was detected among treatments and time 0 (**Fig. 13**). Specifically, the IAA-dependent fluorescence was localised in the vascular cylinder as well as in the cortical region. At day 6, cuttings treated with IBA (**Fig. 14A, B and C**) or 5-BDPU (**Fig. 14D, E and F**) exhibited a similar IAA distribution characterised by a staining scattered throughout the cross section but more intense in the vascular cylinder than in the cortical area. On the other hand, when cuttings were exposed to the mixture of IBA plus 5-BDPU a particular pattern was visualized by IAA immunolocalisation (**Fig. 14G, H and I**). In fact, cross sections from this condition showed localised IAA signal in discrete nodule-like structures around the resin canals (white arrowheads in **fig. 14H**). No major changes in auxin distribution were detected at day 10 when comparing the labelling patterns obtained with IBA, 5-BDPU and IBA plus 5-BDPU (**Fig. 15**). In fact, IAA was homogenously present scattered throughout the cross sections from all the tested conditions.

Effect of BDPUs on *PrSCL1* and *PrSHR* expression



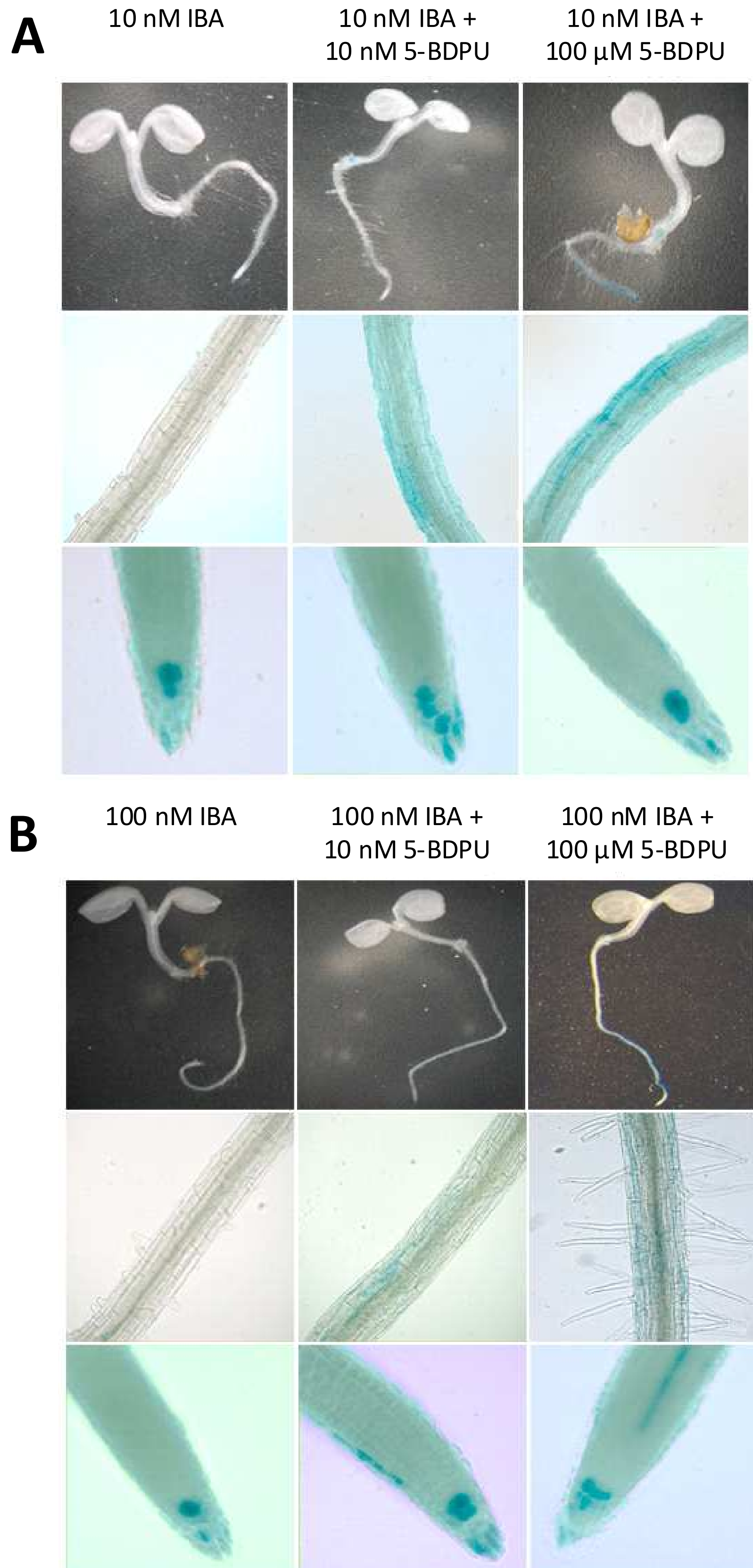


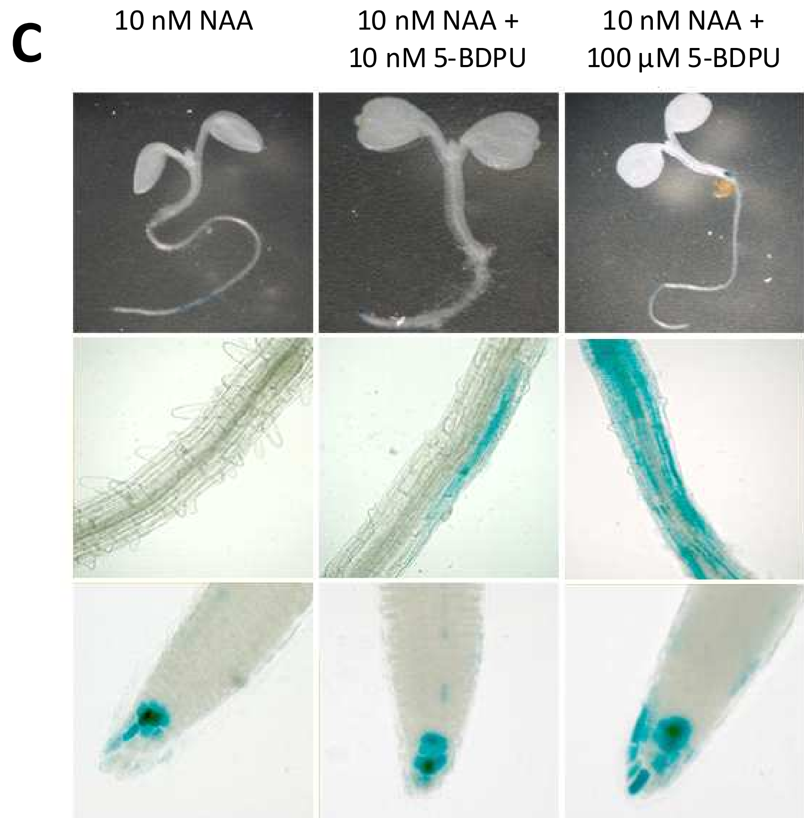
◄ **Fig. 16**

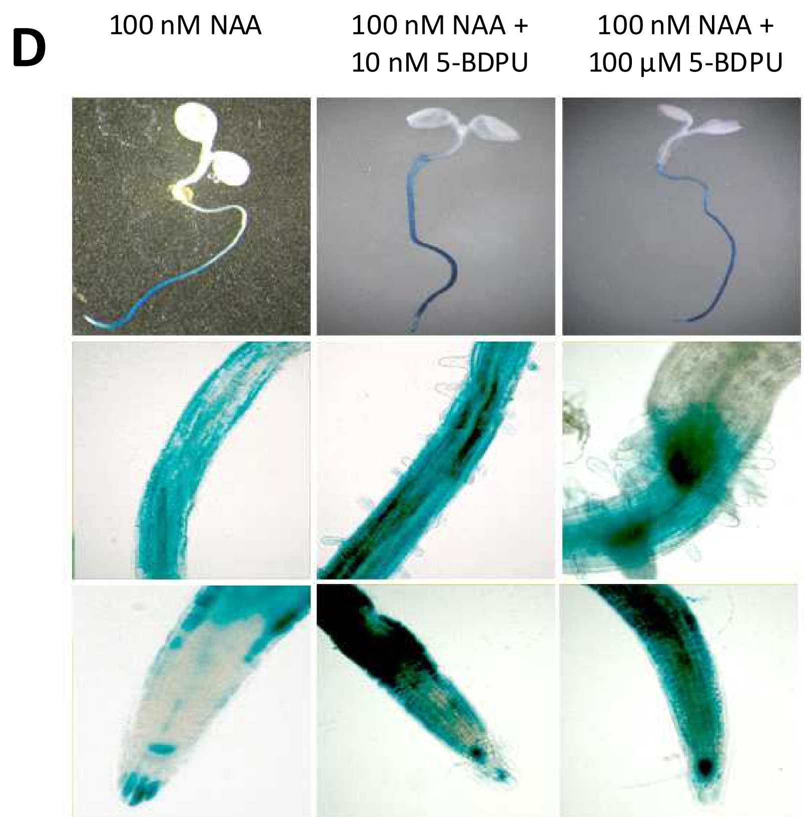
Real-time PCR of *PrSCL1* (A) and *PrSHR* (B) expression in hypocotyl cuttings from 21-day-old *Pinus radiata* seedlings following 24 h-exposure to different concentrations of IBA and BDPUs during adventitious root formation. Quantitative RT-PCR was performed using RNA extracted from the base of cuttings treated for 24 hours with IBA or IBA plus BDPUs. Results are expressed as mean values of relative expression to time 0 ± SE from two biological replicates. Asterisks indicate that the increase of *PrSCL1* gene expression in the presence of 10 µM IBA and 1 µM IBA plus 10 µM BDPUs was significant at $p<0.01$ (Student's *t* test) when compared to that obtained in the presence of 1 µM IBA. No significant difference was detected among treatments and controls for *PrSHR* gene expression.

In order to approach the mode of action of BDPUs and their possible interaction with the auxin signalling pathways, the expression of two genes involved in the early stages of root initiation in *Pinus*, *P. radiata SCARECROW-LIKE1* gene (*PrSCL1*) and a *P. radiata SHORT-ROOT* gene (*PrSHR*) was analysed in response to 24 h-treatment with IBA and IBA plus BDPUs. Maximum mRNA levels of both genes had been measured at this stage (Sánchez *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. Both 5-BDPU and 6-BDPU increase *PrSCL1* mRNA levels in the presence of IBA (**Fig. 16A**), the level of transcripts being significantly higher $p<0.01$ than the ones measured in the presence of auxin alone at the same concentration. The expression of a *PrSHR* was not significantly affected in the presence of both BDPUs alone or combined with auxin (**Fig. 16B**).

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




C
10 nM NAA
10 nM NAA +
10 nM 5-BDPU
10 nM NAA +
100 µM 5-BDPU


D
100 nM NAA
100 nM NAA +
10 nM 5-BDPU
100 nM NAA +
100 µM 5-BDPU

◄ **Fig. 17**

Histochemical localization of GUS activity in 4-day-old transgenic *DR5::GUS Arabidopsis* seedlings following exposure to different concentration of 5-BDPU and to different concentration of auxin, IBA (A and B) or NAA (C and D). Panels show whole seedling, part of the primary root and root tip (from the top to the bottom of the panels). Experiments were repeated three times and representative phenotypes are shown (n = 30).

The synthetic auxin response reporter construct known as *DR5::GUS* is an important tool to localize regions of auxin responsiveness. This construct consists of a minimal cauliflower mosaic virus (CaMVS) 35S promoter placed upstream to seven-copy tandem direct repeats of the ARF-binding site from the soybean G3 promoter fused to the β-glucuronidase (GUS) reporter gene (Ulmasov *et al.*, 1997). Hence, a GUS-dependent staining in transgenic *DR5::GUS Arabidopsis thaliana* plants allows the visualisation of auxin distribution in plant tissues. To test whether BDPUs were able to affect GUS staining in transgenic *DR5::GUS Arabidopsis* plants, we exposed 4-day-old seedlings to 5-BDPU at different concentrations alone or in combination with different concentrations of IBA or NAA. Similarly to *hormone-free* condition, staining occurred at the tips of the cotyledons tips in specific plants and at the root tips in all plants tested when 4-day-old seedlings were treated with 5-BDPU alone, at all the tested 5-BDPU concentrations (data not shown). Intriguingly, when 5-BDPU was simultaneously supplied with auxin, the GUS activity in *DR5::GUS* seedlings increased compared to the control seedlings (auxin alone). As a matter of fact, slight blue colour occurred throughout the primary roots following exposure to 10 or 100 µM 5-BDPU plus 10 or 100 nM IBA, whereas it lacked in primary roots of seedlings treated with the same concentrations of IBA alone (**Figure 17A and B**). NAA induced very high GUS expression when compared to the results observed in the presence of exogenous IBA. All the mixtures made by 5-BDPU and NAA induced stronger GUS activity in the primary root than did the same NAA treatment alone (**Figure 17C and D**). The GUS staining was strongest after 100 µM 5-BDPU plus 100 µM NAA treatment, since GUS activity was clearly visible beyond the root-hypocotyl junction (**Figure 17D**).

BDPUs and cytokinin signalling pathway

In order to verify if BDPUs could interact with cytokinins, we investigated the ability of these compounds to interact with either the *Arabidopsis* cytokinin receptor CRE1/AHK4 or the early cytokinin response gene *ARR5*. Since we assumed a similar adjuvant activity for either 5-BDPU or 6-BDPU, some experiments have been conducted by using only 5-BDPU.

Effects of BDPUs on the activity of cytokinin receptor CRE1/AHK4

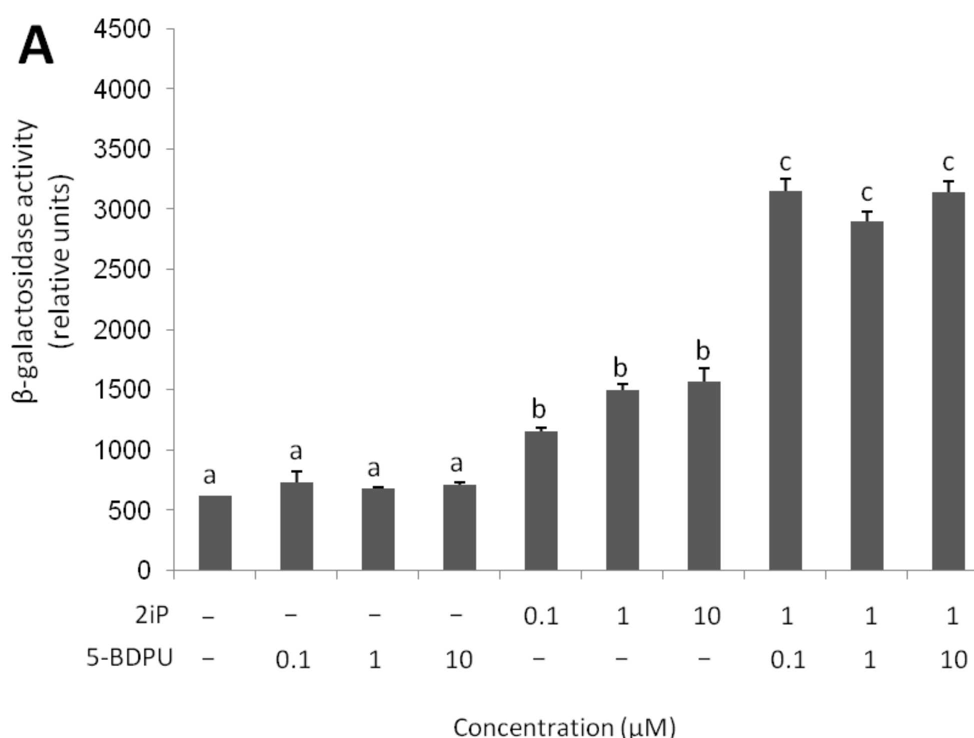


Table 21A - ANOVA of the effect of 2iP, 5-BDPU and their interaction on the induction of β-galactosidase activity in *E. coli* cells. Presented are degrees of freedom (Df), total sums of squares (SS), mean square (MS) for factors and residuals, and *F* and *P* values of the two factors and their interaction.

Source	Df	SS	MS	*F*	*P*
2iP	3	0.05028	0.016760	688.42	< 0.001
5-BDPU	3	0.00352	0.001173	48.19	< 0.001
2iP x 5-BDPU	3	0.00162	0.000540	22.18	< 0.001
Residuals	50	0.00122	0.000024		

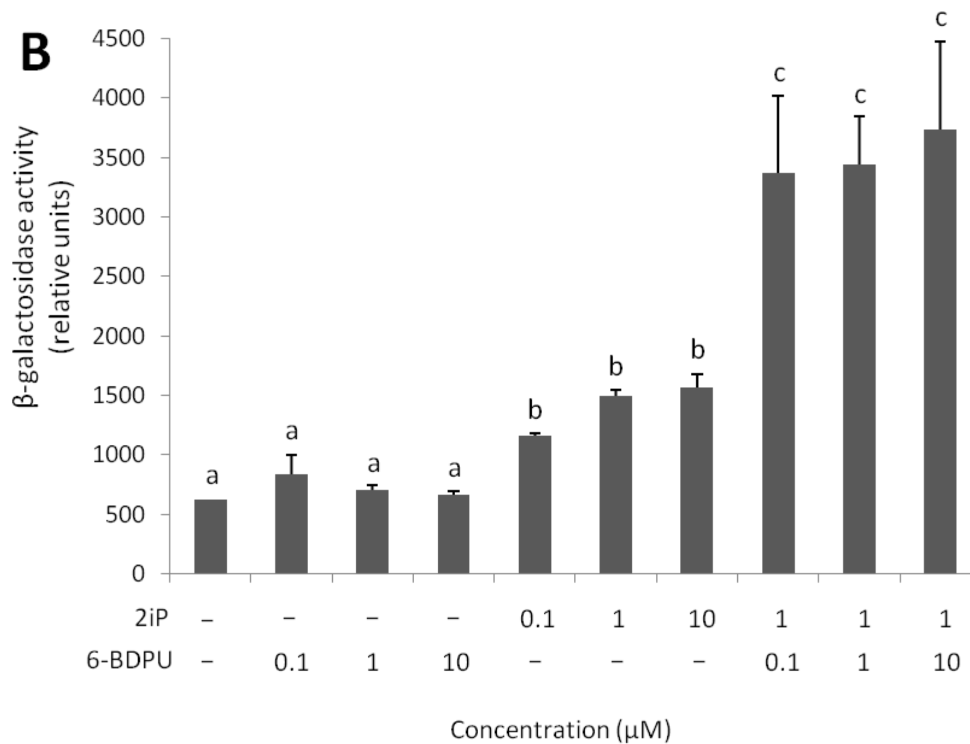


Table 21B - ANOVA of the effect of 2iP, 6-BDPU and their interaction on the induction of β-galactosidase activity in *E. coli* cells. Presented are degrees of freedom (Df), total sums of squares (SS), mean square (MS) for factors and residuals, and *F* and *P* values of the two factors and their interaction.

Source	Df	SS	MS	*F*	*P*
2iP	3	0.007042	0.0023472	340.198	< 0.001
6-BDPU	3	0.000524	0.0001746	25.302	< 0.001
2iP x 6-BDPU	3	0.000197	0.0000656	9.511	< 0.001
Residuals	50	0.000345	0.0000069		

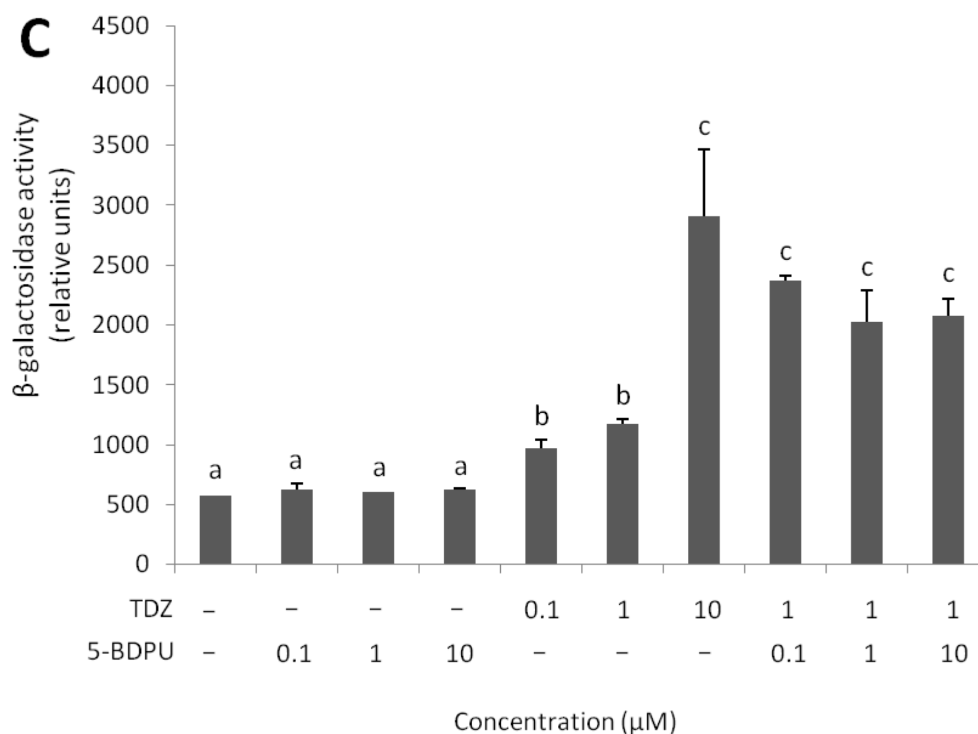


Table 21C - ANOVA of the effect of TDZ, 5-BDPU and their interaction on the induction of β-galactosidase activity in *E. coli* cells. Presented are degrees of freedom (Df), total sums of squares (SS), mean square (MS) for factors and residuals, and *F* and *P* values of the two factors and their interaction.

Source	Df	SS	MS	*F*	*P*
TDZ	3	0.007118	0.0023728	583.55	< 0.001
5-BDPU	3	0.000340	0.0001133	27.85	< 0.001
TDZ x 5-BDPU	3	0.000134	0.0000448	11.01	< 0.001
Residuals	49	0.000199	0.0000041		

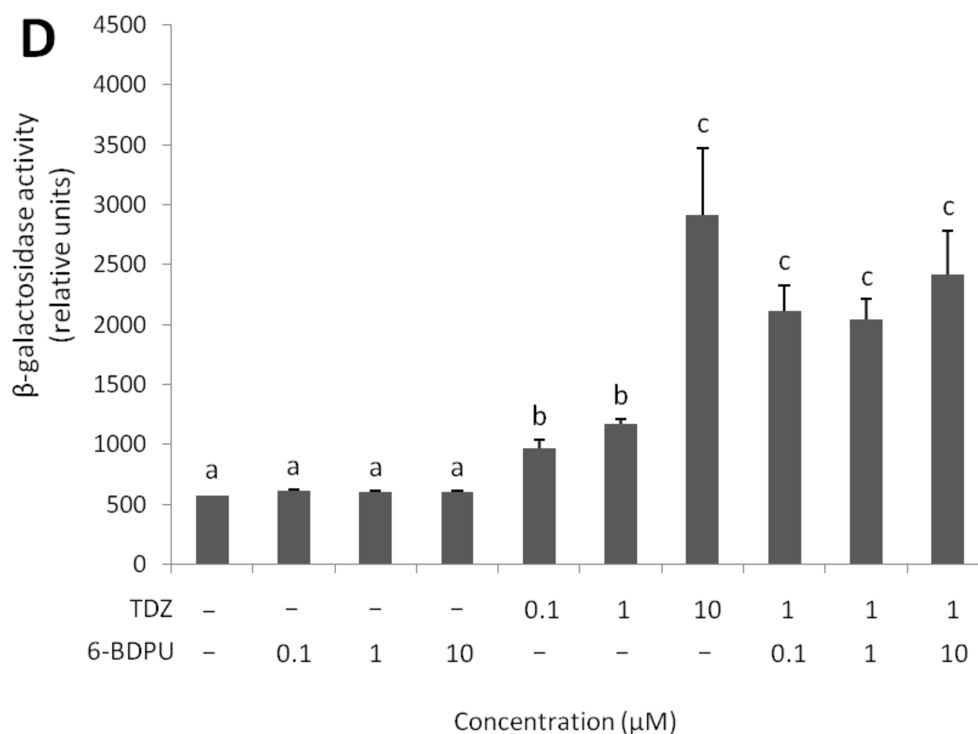


Table 21D - ANOVA of the effect of TDZ, 6-BDPU and their interaction on the induction of β-galactosidase activity in *E. coli* cells. Presented are degrees of freedom (Df), total sums of squares (SS), mean square (MS) for factors and residuals, and *F* and *P* values of the two factors and their interaction.

Source	Df	SS	MS	*F*	*P*
TDZ	3	0.0004387	1.462e-04	619.962	< 0.001
6-BDPU	3	0.0000164	5.460e-06	23.131	< 0.001
TDZ x 6-BDPU	3	0.0000068	2.270e-06	9.607	< 0.001
Residuals	49	0.0000116	2.400e-07		

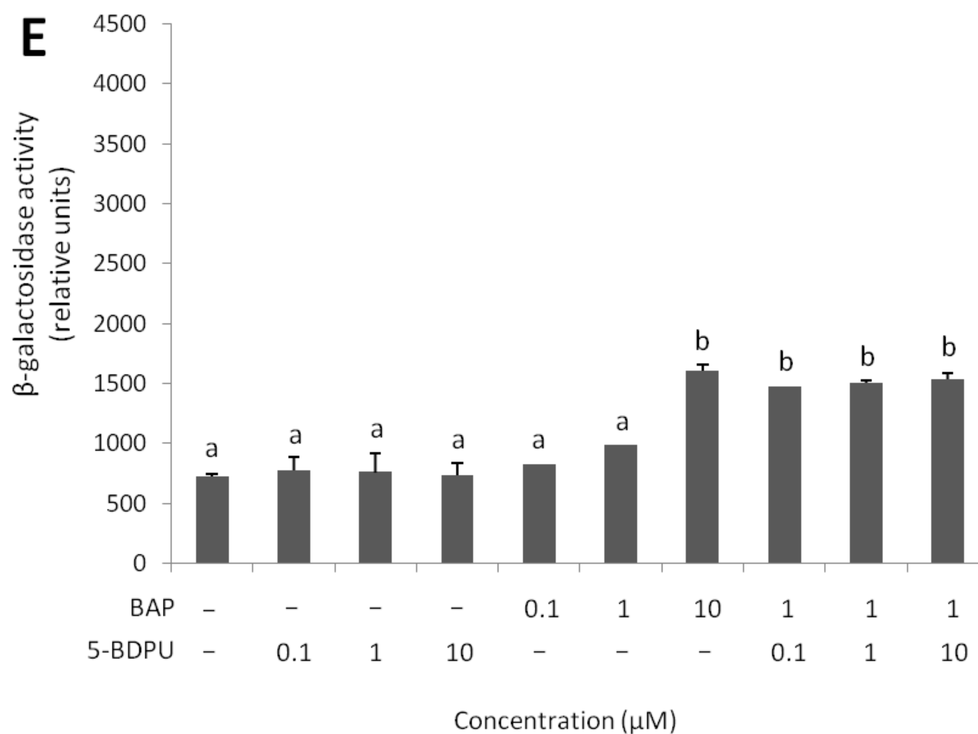


Table 21E - ANOVA of the effect of BAP, 5-BDPU and their interaction on the induction of β-galactosidase activity in *E. coli* cells. Presented are degrees of freedom (Df), total sums of squares (SS), mean square (MS) for factors and residuals, and *F* and *P* values of the two factors and their interaction.

Source	Df	SS	MS	*F*	*P*
BAP	3	5.813	1.9376	122.886	< 0.001
5-BDPU	3	0.444	0.1480	9.388	< 0.001
BAP x 5-BDPU	3	0.378	0.1261	7.999	< 0.001
Residuals	50	0.788	0.0158		

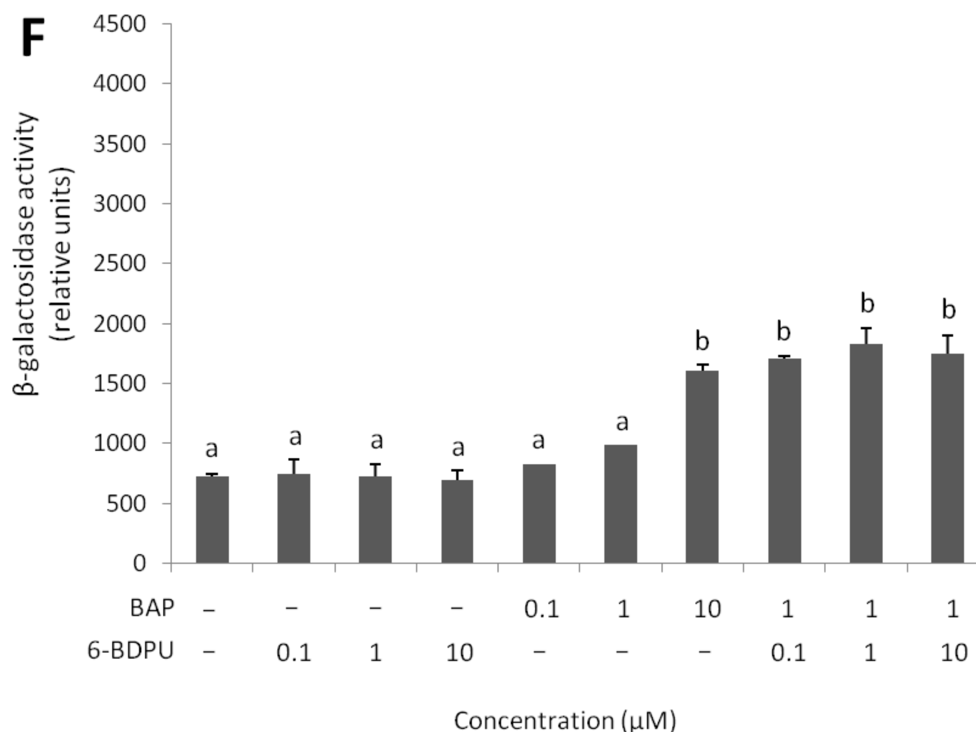


Table 21F - ANOVA of the effect of BAP, 6-BDPU and their interaction on the induction of β-galactosidase activity in *E. coli* cells. Presented are degrees of freedom (Df), total sums of squares (SS), mean square (MS) for factors and residuals, and *F* and *P* values of the two factors and their interaction.

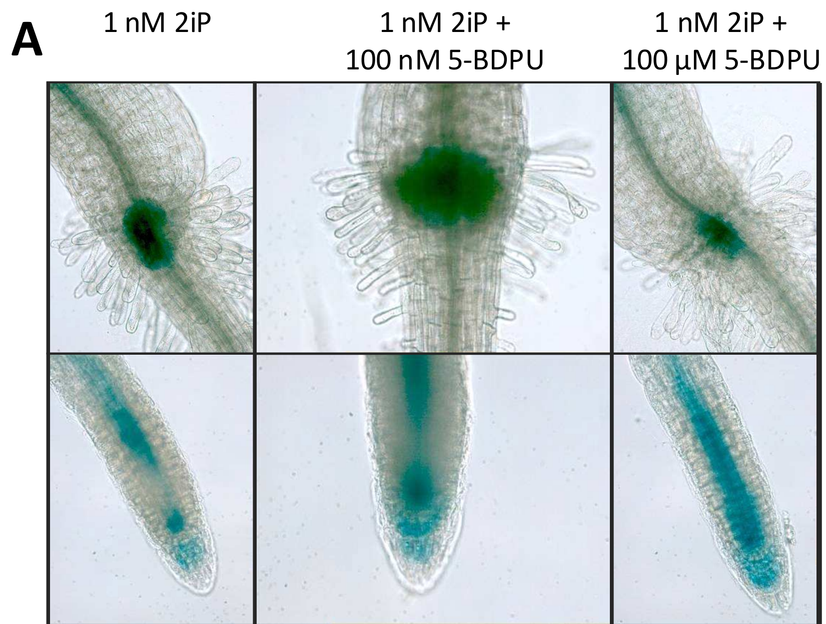
Source	Df	SS	MS	*F*	*P*
BAP	3	8.183	2.7278	176.62	< 0.001
6-BDPU	3	0.690	0.2300	14.89	< 0.001
BAP x 6-BDPU	3	0.836	0.2786	18.04	< 0.001
Residuals	50	0.772	0.0154		

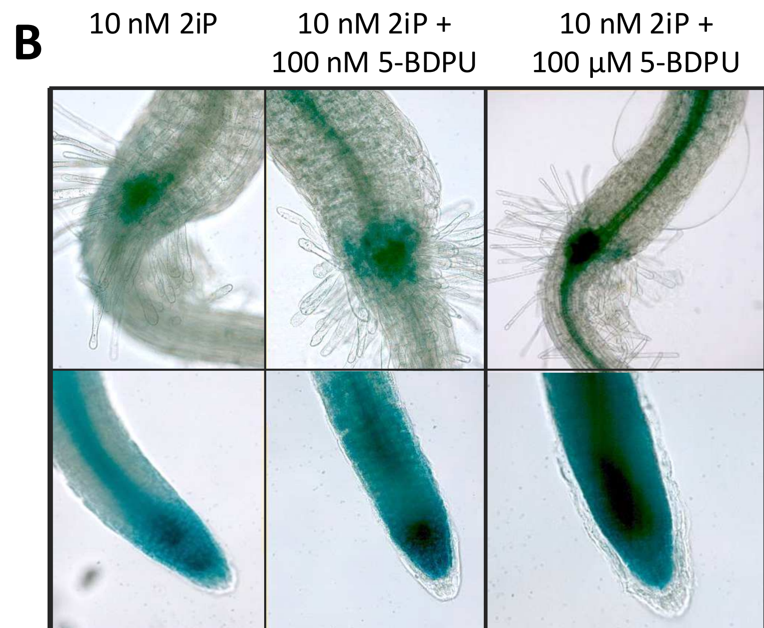
Fig. 18

Comparison of the sensitivity of CRE1/AHK4 to different concentrations of BDPUs , cytokinins (2iP, TDZ and BAP) and mixtures of cytokinins and BDPUs in a bacterial reporter assay. The compounds were tested at the indicated concentrations. Error bars show SE (n=2). Bars with different letters are significantly different at $p<0.01$ level by Tukey's HSD test.

In order to analyze the capacity of 5-BDPU and 6-BDPU to interact with the cytokinin signalling pathway, we studied the interactions of BDPUs, cytokinins (2iP, TDZ and BAP) and mixtures of BDPUs and cytokinins with the CRE1/AHK4 receptor of *Arabidopsis*. For this, we employed transformed *Escherichia coli* strain expressing the cytokinin receptor CRE1/AHK4 and the cytokinin-activated reporter gene *cps::lacZ*. Therefore, the activation of the signalling pathway can be easily monitored by measuring β-galactosidase activity of *E. coli* cells. CRE1/AHK4 is a primary receptor that directly binds a variety of natural and synthetic cytokinins (*e.g.*, not only N^6-substituted aminopurines such as 2iP, BAP but also urea derivatives such as TDZ). This cytokinin receptor was the only one we used in this study rather than either of the other two *Arabidopsis* cytokinin receptors, AHK2 and AHK3, because it has been reported that CRE1/AHK4 provokes a several fold higher response in *E. coli* than AHK3, even if they showed different ligand specificity (Spíchal *et al.*, 2004). Data presented in **Fig.184** show that neither 5-BDPU nor 6-BDPU was able alone to activate the receptor, at any of the tested concentrations (0.1, 1 and 10 μM), confirming that BDPUs did not show any cytokinin-like activity *per se*. As expected, all the tested cytokinins activated the CRE1/AHK4-mediated signalling pathway in a dose-dependent manner. Intriguingly, in the simultaneous presence of one cytokinin supplemented at 1 μM plus either 5-BDPU or 6-BDPU supplemented at different concentrations, the receptor activation was several fold greater than that obtained in the presence of the same concentration of the same cytokinin alone. As shown in **Fig. 18A and B**, the mixtures made by either 5-BDPU or 6-BDPU plus 1 μM 2iP were able to trigger a response that was greater than that observed by 1 μM 2iP alone. In addition, the response obtained with these mixtures was higher than that caused by a concentration 10-fold greater (10 μM) of 2iP alone. A similar activity with AHK4/CRE1 was also found for the mixtures made by BDPUs plus 1 μM BAP or TDZ (**Fig. 18C, D, E and F**). Moreover, the activation of the receptor as a result of treatments with combinations of cytokinin plus BDPUs appeared to be independent of the dose of BDPUs. The two-way ANOVA indicated a significant interaction among all the tested cytokinins and 5-BDPU or 6-BDPU (**Table 21A-F**).

Effect of BDPUs on GUS activity in P_{ARR5}*:GUS* transgenic plants





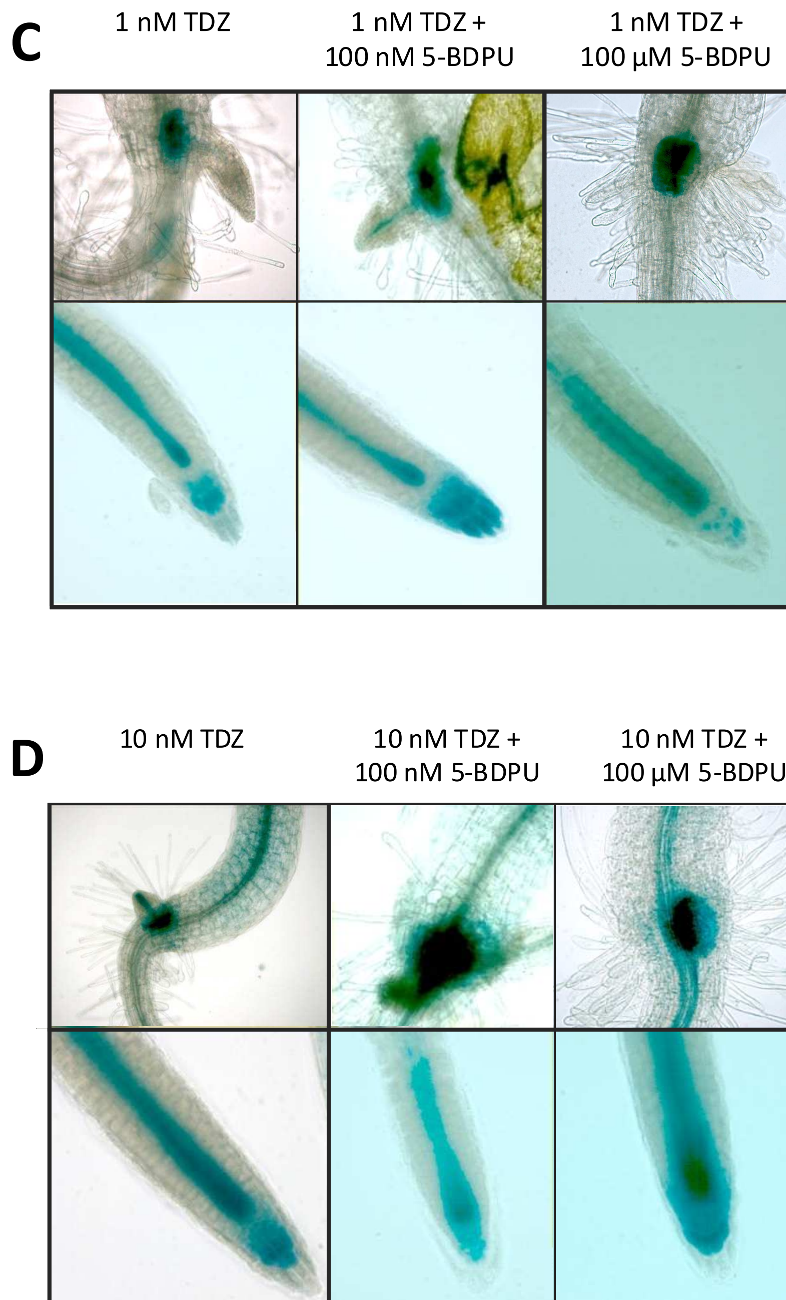


Fig. 19

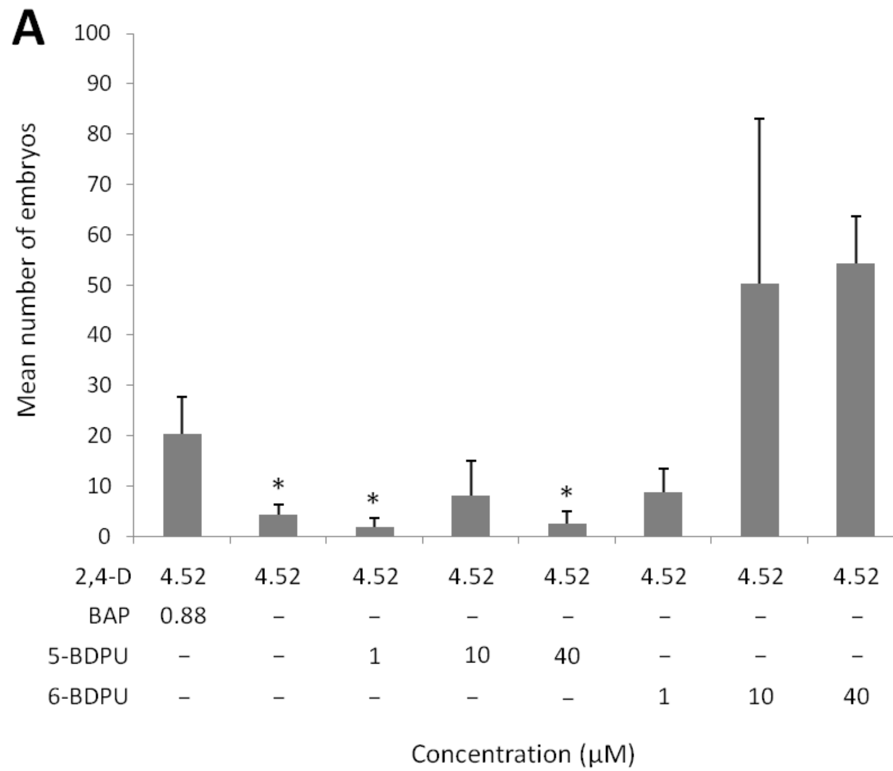
Histochemical localization of GUS activity in 4-day-old transgenic P_{ARR5}*:GUS Arabidopsis* seedlings following exposure to different concentration of 5-BDPU and to different concentration of cytokinin, 2iP (A and B) or TDZ (C and D). Panels show hypocotyl-root junction and root tip (from the top to the bottom of the panels). Experiments were repeated three times and representative phenotypes are shown (n = 30).

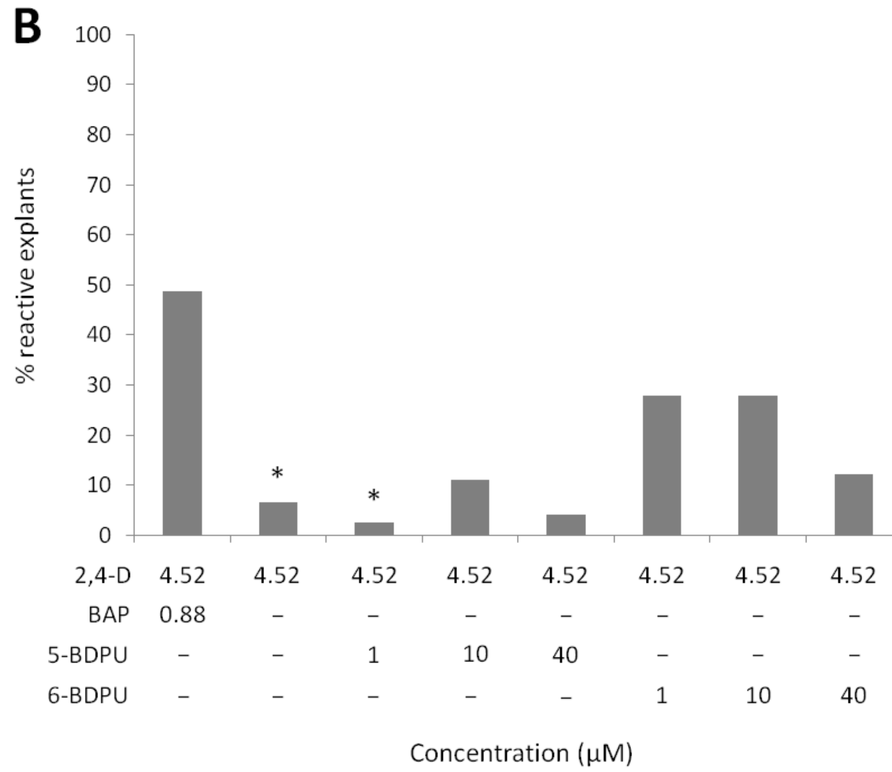
Following our finding that BDPUs somehow interact with cytokinins for binding to cytokinin receptor CRE1/AHK4, we tried to determine whether 5-BDPU interacts with cytokinins in cytokinin signal transduction *in planta*. For this, we used transgenic plants

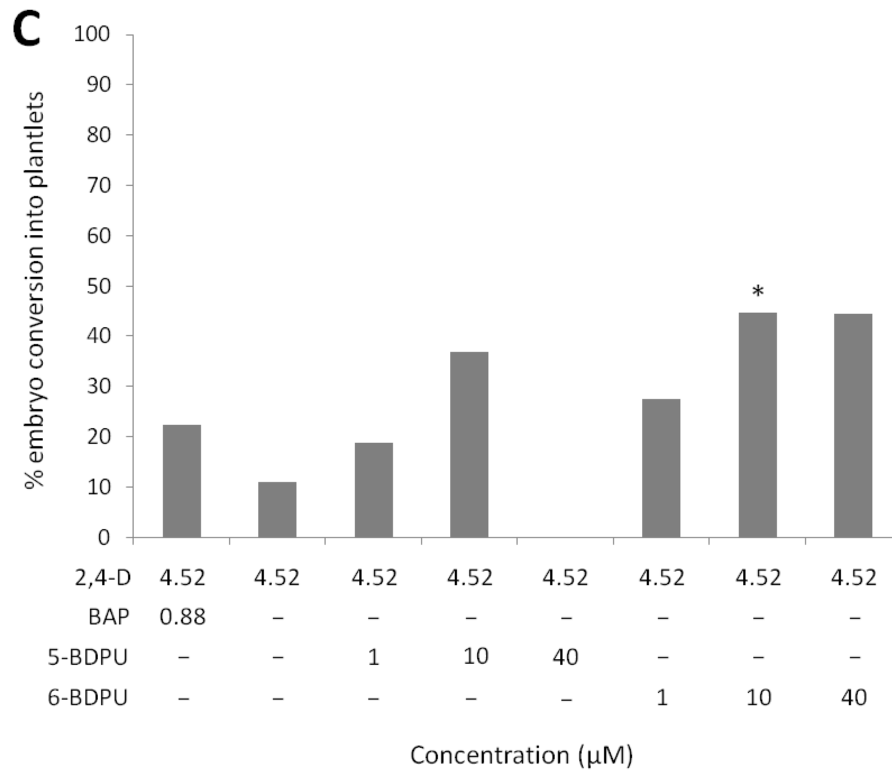
harbouring T-DNA containing a fusion of 1.6 kb of the *ARR5* promoter region (P_{ARR5}) to a GUS reporter gene (D'Agostino *et al.*, 2000). *ARR5* displays properties of cytokinin primary-response gene. As a result, the response of the P_{ARR5}*:GUS* reporter gene is rapidly upregulated by cytokinins and can be monitored by histochemical analysis of GUS activity. We recorded the spatial domains of P_{ARR5}*:GUS* gene activation in 4-day-old *Arabidopsis* seedlings following treatments with 5-BDPU plus 2iP or TDZ. GUS staining obtained when seedlings were treated with 5-BDPU alone was similar to that observed in *hormone-free* condition, at all the 5-BDPU tested concentrations. In fact, the most prominent GUS expression was seen in the root cap columella cells, the root vasculature and shoot meristem regions (data not shown). **Figure 19** shows the expression of P_{ARR5}*:GUS* in root tips and hypocotyl-root junctions following exposure to the mixtures. Seedlings treated with combinations of 100 nM or 100 µM 5-BDPU plus the lowest concentration of 2iP or TDZ were no darker than controls (1 nM 2iP or TDZ alone) (**Fig. 19A and C**). Conversely, the blue staining caused by P_{ARR5}*:GUS* expression following treatment by 10 nM 2iP or TDZ plus 100 nM or 100 µM 5-BDPU increased compared to the control (10 nM 2iP or TDZ alone) (**Fig. 19B and D**). Moreover, GUS activity caused by treatment with these mixtures appeared to be affected by the concentration of 5-BDPU. In fact, when seedlings were exposed to 10 nM 2iP plus 100 nM or 100 µM 5-BDPU, intense GUS staining was observed in the hypocotyl-root junction, root tip and inside and outside the root vasculature, whereas treatment with 10 nM 2iP alone caused P_{ARR5}*:GUS* expression in the hypocotyl-root junction, root tip and inside the root vasculature only (**Fig. 19B**). In addition, in the presence of 10 nM 2iP plus 100 µM 5-BDPU, the staining was even visible in the hypocotyl vasculature. Similarly, treatment with 10 nM TDZ alone induced less GUS activity in the root tip than that did the simultaneous presence of 100 nM or 100 µM 5-BDPU and 10 nM TDZ, reaching a maximum in the presence of the highest 5-BDPU concentration (**Fig. 19D**).

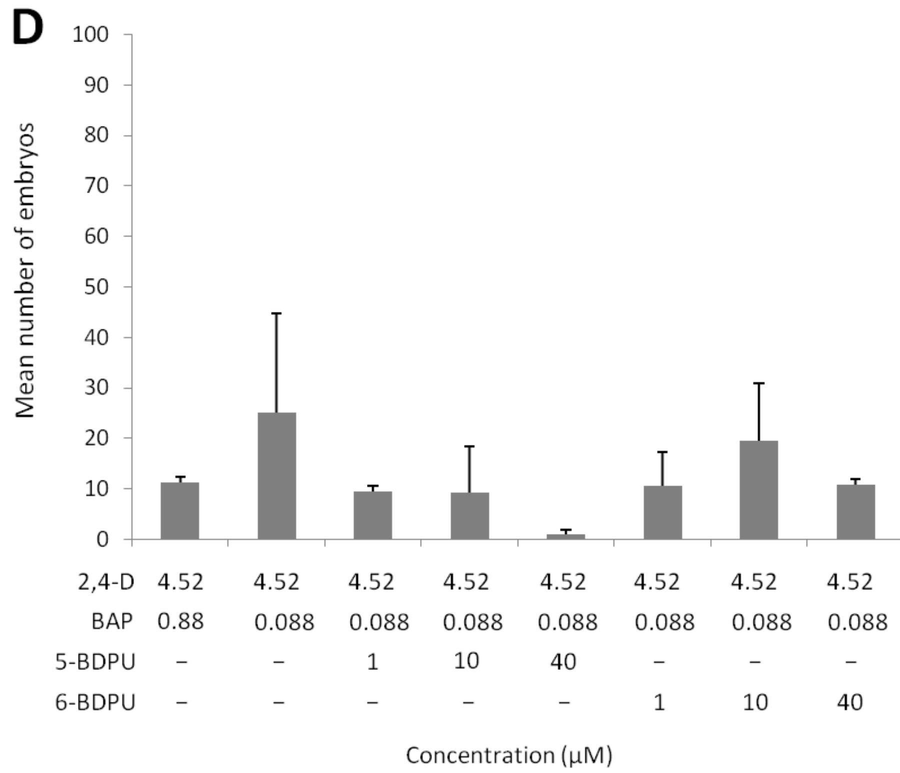
Somatic embryogenesis

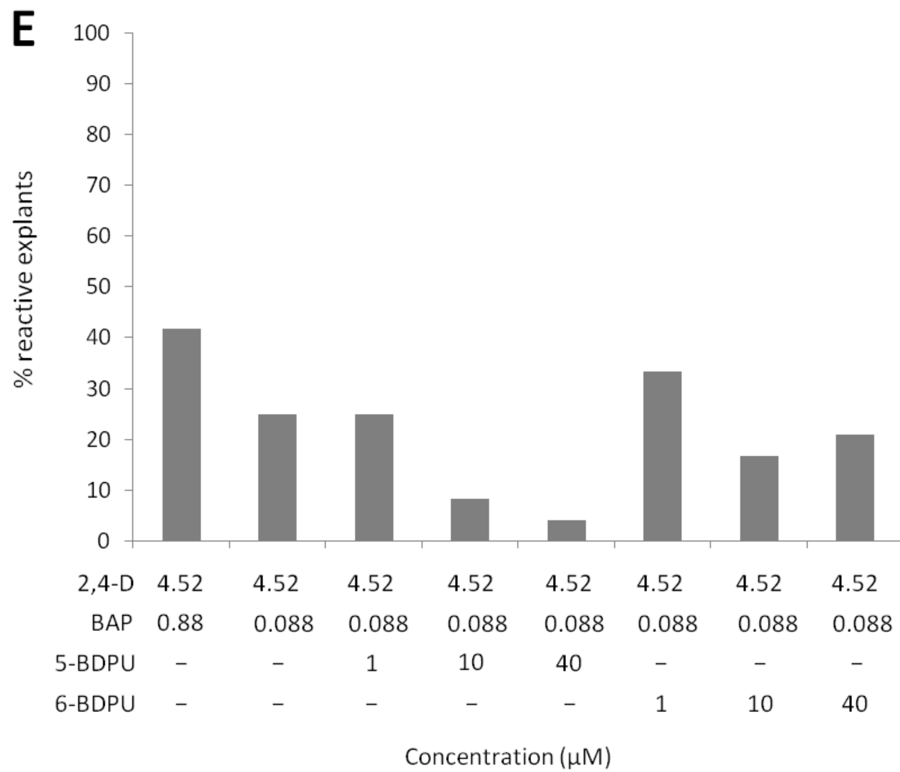
Effect of BDPUs on somatic embryogenesis of *Medicago truncatula*




B
% reactive explants
100
90
80
70
60
50
40
30
20
10
0
*
*
2,4-D 4.52 4.52 4.52 4.52 4.52 4.52 4.52 4.52
BAP 0.88 – – – – – – –
5-BDPU – – 1 10 40 – – –
6-BDPU – – – – – 1 10 40
Concentration (µM)


C
% embryo conversion into plantlets
100
90
80
70
60
50
40
30
20
10
0
*
2,4-D 4.52 4.52 4.52 4.52 4.52 4.52 4.52 4.52
BAP 0.88 – – – – – – –
5-BDPU – – 1 10 40 – – –
6-BDPU – – – – – 1 10 40
Concentration (µM)


D
Mean number of embryos
100
90
80
70
60
50
40
30
20
10
0
2,4-D 4.52 4.52 4.52 4.52 4.52 4.52 4.52 4.52
BAP 0.88 0.088 0.088 0.088 0.088 0.088 0.088 0.088
5-BDPU – – 1 10 40 – – –
6-BDPU – – – – – 1 10 40
Concentration (µM)


E
% reactive explants
100
90
80
70
60
50
40
30
20
10
0
2,4-D 4.52 4.52 4.52 4.52 4.52 4.52 4.52 4.52
BAP 0.88 0.088 0.088 0.088 0.088 0.088 0.088 0.088
5-BDPU – – 1 10 40 – – –
6-BDPU – – – – – 1 10 40
Concentration (µM)

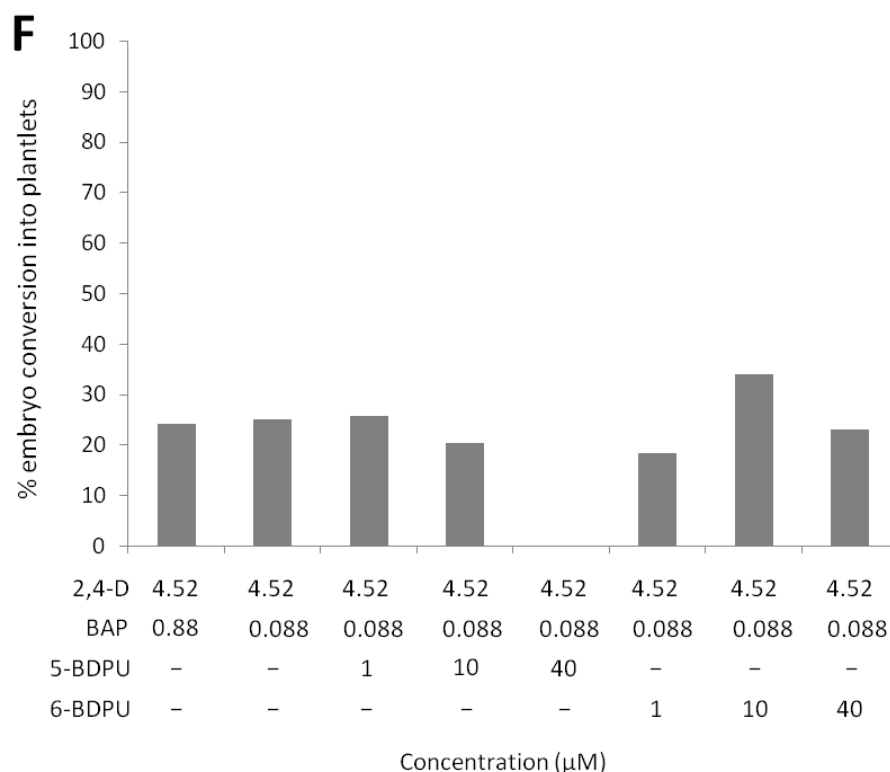


Figure 20

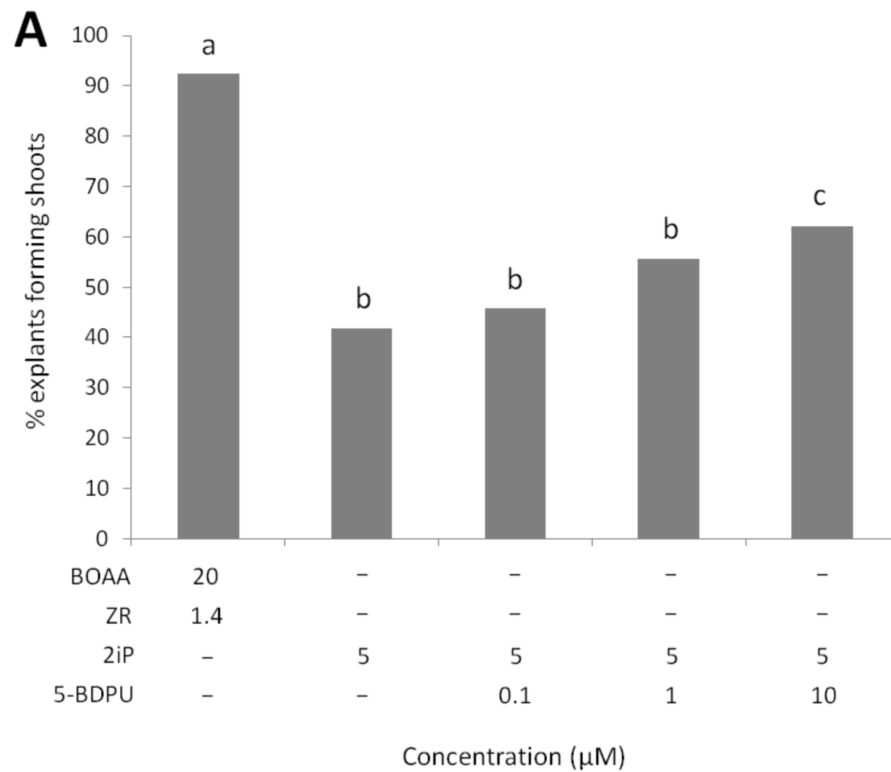
Effect of BDPUs on somatic embryogenesis of *Medicago truncatula* line R108 leaf explants evaluated in terms of mean number of embryos per explant (A and D), percentage of explants which form embryos (B and E) and percentage of embryo conversion into normal plantlets (C and F). The mixture made by 4.52 µM 2,4-D plus 0.88 µM BAP was used as a positive control for embryo induction and development. Results obtained following exposure to 4.52 µM 2,4-D alone or in combination with different concentrations of BDPUs are reported in charts A, B and C. Results obtained following exposure to binary mixture consisting of 4.52 µM 2,4-D plus ten-fold less concentrated (0.088 µM) BAP or ternary mixtures consisting of 4.52 µM 2,4-D plus 0.088 µM BAP plus different concentrations of BDPUs are reported in charts D, E, and F. Asterisk indicated significant difference between treatments and the positive control (4.52 µM 2,4-D plus 0.88 µM BAP) at $p \leq 0.05$ level by WMW test. Error bars in panels A and D show SE (n=2).

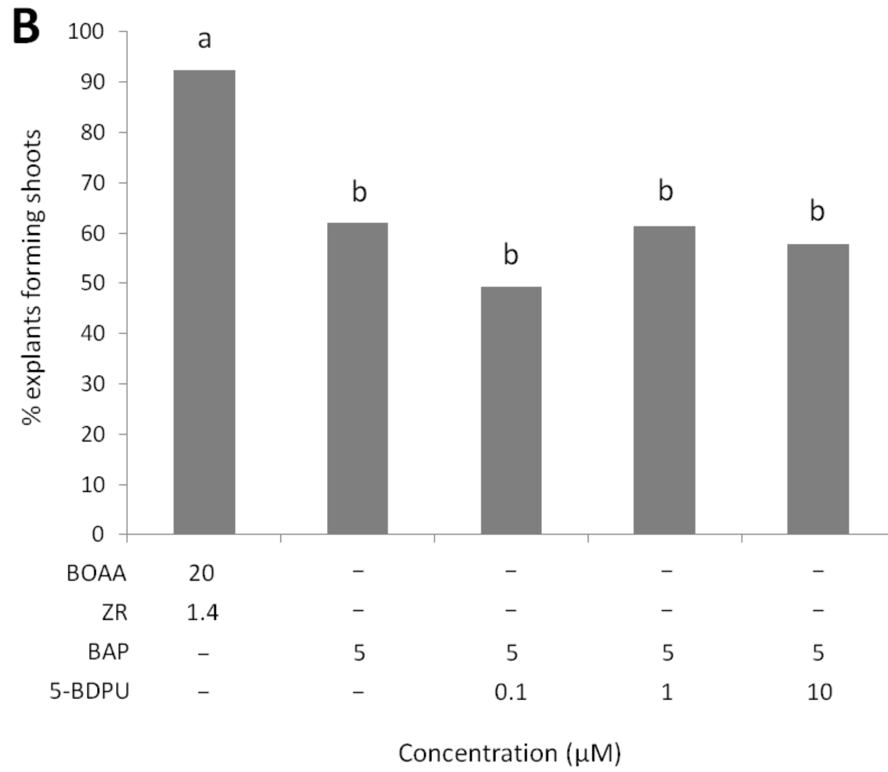
In order to investigate the effect of BDPUs on somatic embryogenesis, we tested their hypothetical adjuvant activity in this physiological process by using *Medicago truncatula* line R108 leaf explants which readily form embryos following simultaneous exposure to 4.52 µM 2,4-D and 0.88 µM BAP. For this, leaf explants were exposed to different concentrations of BDPUs alone or binary mixtures made by 0.88 µM BAP or 4.52 µM 2,4-D plus different concentrations of BDPUs. We also used ternary mixtures made by ten-fold less concentrated (0.088 µM) BAP plus 4.52 µM 2,4-D plus different

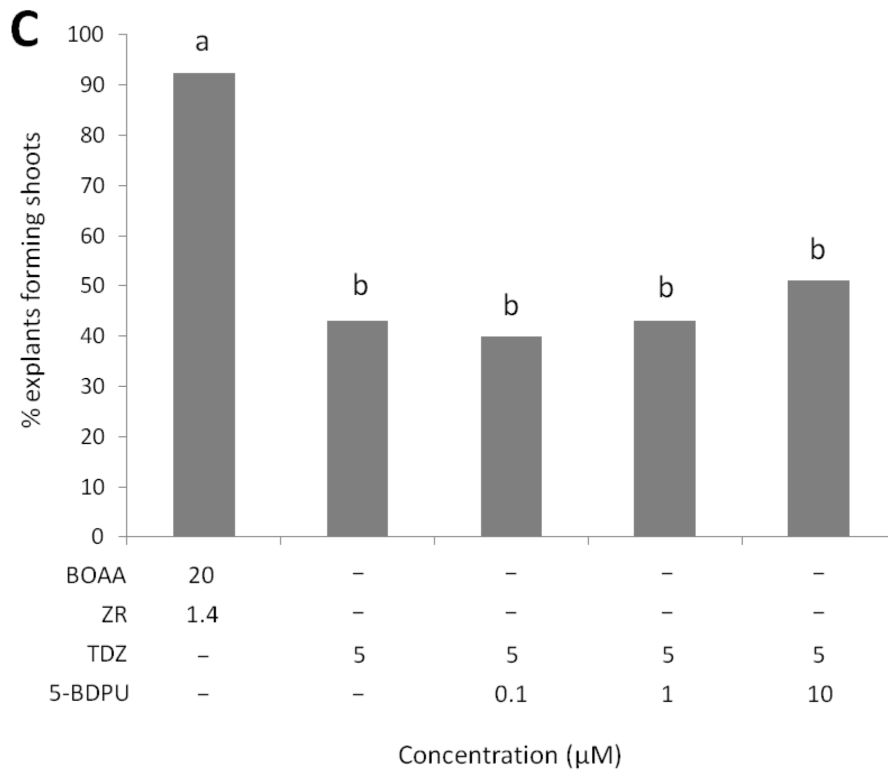
concentrations of BDPUs. Similarly to *hormone-free* condition, leaf explants did not react when incubated with BDPUs alone or in combination with 0.88 µM BAP (data not shown). **Figures 20A, B and C** show the effect of combinations of 4.52 µM 2,4-D plus BDPUs. In the presence of 4.52 µM 2,4-D alone, either the embryo mean number or the percentage of explants which form embryos was significantly lower than that recorded in the presence of the mixture made by 4.52 µM 2,4-D plus 0.88 µM, confirming that both auxin and cytokinin are essential to induce embryo formation (**Fig. 20A and B**). On the other hand, the presence of 4.52 µM 2,4-D alone did not significantly affect the percentage of embryo conversion into plantlets when compared to the positive control (4.52 µM 2,4-D plus 0.88 µM) (**Fig. 20C**). Similarly to treatment with 2,4-D alone, the mean number of embryos obtained in the presence of the mixtures made by 2,4-D plus 1 or 40 µM 5-BDPU was significantly lower than that recorded in the presence of the control condition (4.52 µM 2,4-D plus 0.88 µM). Conversely, exposure of leaf explants to the combinations of 2,4-D plus 10 µM 5-BDPU or 1, 10 or 40 µM 6-BDPU caused a similar effect to that recorded with the control condition, since the mean numbers of embryos were not significantly different (**Fig. 20A**). Among the tested mixtures made by 2,4-D plus BPDUs, only 2,4-D plus 1 µM 5-BDPU determined a significant decrease of the percentage of explants forming embryos when compared to that recorded with the positive control (**Fig. 20B**). When the percentage of embryo conversion into plantlets was considered, only the simultaneous presence of 2,4-D plus 10 µM 6-BDPU induced a significant increase if compared to the result obtained with the positive control (**Fig 20C**). No significant differences have been detected following exposure to combinations of 4.52 µM 2,4-D plus 0.088 µM BAP plus 1, 10 or 40 µM BDPUs when compared to the controls (4.52 µM 2,4-D plus 0.88 µM or 4.52 µM 2,4-D plus 0.088 µM) for any of the three considered parameters (**Fig. 20D, E and F**).

Shoot regeneration

Effect of BDPUs and cytokinins on shoot regeneration of *Lycopersicon esculentum*




B
100
90
80
70
60
50
40
30
20
10
0
a
b
b
b
b
% explants forming shoots
BOAA 20 – – – –
ZR 1.4 – – – –
BAP – 5 5 5 5
5-BDPU – – 0.1 1 10
Concentration (µM)


C
100
90
80
70
60
50
40
30
20
10
0
a
b
b
b
b
% explants forming shoots
BOAA 20 – – – –
ZR 1.4 – – – –
TDZ – 5 5 5 5
5-BDPU – – 0.1 1 10
Concentration (µM)

◄ **Fig. 21**
Effect of combinations of different concentrations of 5-BDPU plus 5 µM 2iP (A), BAP (B) or TDZ (C) on shoot regeneration in the tomato cotyledon bioassay. The mixture made by 20 µM BOAA plus 1.4 µM ZR was used as a positive control for tomato shoot regeneration. Results are expressed as percentage of explants forming shoots. Bars with different letters are significantly different at *p<0.05* level by WMW test.

To test whether BDPUs were able to interact with cytokinins during shoot regeneration, we exposed tomato cotyledons to mixtures made by 5 µM cytokinin plus different concentrations of 5-BDPU. To validate the shoot regeneration efficiency of tomato cotyledons, explants were exposed to combinations of 20 µM BOAA plus 1.4 µM ZR. Data obtained following treatments with 2iP, BAP or TDZ plus 5-BDPU were reported in **figure 21A, B and C,** respectively. Only in the simultaneous presence of 5 µM 2iP and 10 µM 5-BDPU the percentage of explants forming shoots was enhanced, as it was significantly higher than that of the same 2iP concentration alone (**Fig. 21A**). No significant enhancement was detected when cotyledon explants were exposed to the mixtures made by BAP or TDZ and 5-BDPU (**Fig. 21B and C**).

DISCUSSION

Symmetric urea derivatives, such as *N*,*N*'-bis-(2,3-methylenedioxyphenyl)urea (2,3-MDPU) and the *N*,*N*'-bis-(3,4-methylenedioxyphenyl)urea (3,4-MDPU), namely MDPUs, can act either as adventitious rooting adjuvants or as somatic embryogenesis adjuvants (Ricci *et al.*, 2001b; 2003; Carra *et al.*, 2006). These compounds do not show any auxin- or cytokinin-like activity *per se*, rather it seems that they magnify the response to the growth regulator stimulus of naturally competent-to-root or -to-form-somatic-embryos cell niches. When a chemical modification of the aromatic moiety of MDPUs consisting in a replacement of the methylenedioxyphenyl groups by the isostere benzoxazole ones was performed, the two new compounds, 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), namely BDPUs (**Fig. 4**), showed a conserved adventitious rooting enhancement without either auxin- or cytokinin-like activity *per se*, as already demonstrated by the lead compounds (Ricci *et al.*, 2006). These authors hypothesized 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 report new insights about the adjuvant activity of BDPUs in several physiological processes, in which auxins and/or cytokinins are involved, as adventitious rooting, somatic embryogenesis and shoot regeneration. In addition, here we describe our findings regarding the interaction among BDPUs and the cytokinin signalling pathway.

BDPUs and adventitious rooting

The induction of adventitious roots is crucial for vegetative propagation via cuttings and for *in vitro* micropropagation. In horticulture and forestry, the formation of adventitious roots allows the cloning of superior genotypes and is an essential part of breeding programs. However, rooting capacity varies among species and with genotype. Adventitious rooting capacity is generally lower in woody species than in herbaceous ones. 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. Frequently the rooting competence of specific cell types remains unexpressed *in planta*. However, it may be elicited through *in vitro* culture, as a consequence of wounding *per se*, or combined with the action of specific inducers. Auxins are able to consistently enhance adventitious root formation in cuttings. As a matter of fact, improvements in the rooting of difficult to root species have been achieved through exogenous auxin supplementation. Moreover, the type of auxin, concentration or exposure time could affect the process. Nonetheless, there are still many instances of rooting recalcitrance, especially in forest trees and woody horticultural plants. Therefore, 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. Here we describe the results obtained by investigating the action spectrum and the mode of action of BDPUs, which behave as rooting adjuvants.

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. In addition, the mixtures made by BDPUs and IBA were employed to analyse the effect of BDPUs on the adventitious rooting capacity of difficult to root carob and strawberry tree microcuttings. Almost no roots were formed were formed when the hypocotyl cuttings from 21-day-old *P. radiata* seedlings, carob and strawberry tree microcuttings were cultured in *hormone free* (data not shown). Similarly, BDPUs were ineffective when supplemented alone to the pine cuttings, carob and strawberry tree microcuttings (data not shown), whereas they enhanced the adventitious root formation when supplemented to the *A. thaliana* etiolated seedlings at specific concentrations (**Fig. 9A**). In this experimental system, BDPUs could likely interact with endogenous auxin pool triggering adventitious root formation in the *A. thaliana* etiolated seedlings since adventitious roots are formed in the control condition as well (Sorin *et al.*, 2005), while exogenous auxin is required for adventitious root formation in *P. radiata* hypocotyl

cuttings (Sanchéz *et al.*, 2007; Solé *et al.*, 2008; Ricci *et al.*, 2008), microcuttings of carob (Romano *et al.*, 2002) and strawberry tree (Mereti *et al.*, 2002; Brunoni *et al.*, 2012). The enhancement of adventitious rooting in the presence of BDPUs and exogenous auxin depends on the type of auxin and exposure time to the mixture. 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 (**Fig. 9B**) and in the *P. radiata* hypocotyl cuttings (**Table 15A**), confirming the results already reported using apple stem slices test (Ricci *et al.*, 2006). The enhancement of adventitious root formation of *in vitro* micropropagated carob tree microcuttings was only observed following the exogenous supplementation of a mixture composed by BDPUs and IBA for a short time exposure (**Table 16**), suggesting that the exposure time of the treatment IBA plus BDPUs may affect the rooting response as well. Otherwise, we did not record any enhancement of adventitious root formation of *in vitro* micropropagated strawberry tree microcuttings at any of the tested incubation time (**Table 19 and 20**), suggesting that either the assayed mixture made by IBA plus BDPUs or the exposure time of this treatment might not be able to influence the adventitious rooting process in strawberry tree. As a matter of fact, it has been reported that the mixture made by IBA plus 2,3-MDPU supplied for 3 days significantly improved the percentage of rooted microcuttings compared to the control with IBA alone for the same incubation time (Brunoni *et al.*, 2012), suggesting that at least one symmetrical urea derivative belonging to this new category of adventitious rooting adjuvants was effective in enhancing the rooting capacity of strawberry tree microcuttings. The histological time course of adventitious rooting in *P. radiata* hypocotyl cuttings and *in vitro* micropropagated carob tree microcuttings 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 'internal nodules' located near the resin canals, visible after 10 days of culture either in the absence of exogenous auxin (**Fig. 11B**) or in the presence of 10 μM 5-BDPU alone (**Fig. 11H**), 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 'nodules' were still clearly visible after 13 days of treatment in the presence of 5-BDPU (**Fig. 11I**), suggesting that these urea derivatives could interact with the sensitivity of cells naturally competent to respond to auxin stimulus promoting the localisation of cell

divisions and preventing the callus formation. The 'internal nodules' are induced after 6 days of culture (**Fig. 11J**), root primordia and roots are visible after 10 and 13 days of culture, respectively (**Fig. 11K and 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. The presence of these globular-shaped cell division areas could explain the absence of callus formation at the base of the cuttings. Similarly to *Pinus*, cross sections of the base of carob microcuttings exposed for 3 days to 10 µM 5-BDPU alone and examined at day 3, 6 and 12 showed dividing cells which did not give rise to root primordia development (**Fig. 12D, E and F**). Nonetheless, we can hypothesize that those cell divisions may be an effect of the presence of 5-BDPU on cambial cells, even in the absence of exogenous auxin. Both the 3 day-treatments with 1 µM IBA alone and in combination with 10 µM 5-BDPU induced root primordia formation which were visible at day 12 (**Fig. 12C and I**). However, root primordia formation was direct and well organized following 3 day-exposure to the mixture made by IBA plus 5-BDPU, whereas a mass of callus tissue from which root primordia originated was detected in cross sections from microcuttings treated with IBA alone. These findings indicate that, in the simultaneous presence of auxin, BDPUs could likely exert their effect on adventitious root formation in carob by stimulating the responsive cells, confirming the results reported above for *Pinus*. In the simultaneous presence of NAA, a stronger auxin than IBA, the rooting enhancement was obtained in the presence of low BDPU concentrations in both the *Arabidopsis* etiolated seedlings (**Fig. 9C**) and in apple stem slice (**Fig. 10**). In the latter experimental system, the rooting enhancement dramatically decreased 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. Moreover, the adventitious rooting enhancement is only observed in the simultaneous presence of the lowest NAA concentration (0.1 µM, ten-fold lower than that of IBA) and 10 µM 5-BDPU in the *Pinus* hypocotyl cuttings (**Table 15B**). The effect of BDPUs on the localised cell division at the root initiation sites, the formation of the 'internal nodules' during the early stages of adventitious root formation (6 days) in *Pinus* cuttings in the presence of low concentration of exogenous auxin, the direct formation of root primordia from cambial derivatives (12 days) in carob microcuttings, following 3 day-exposure to combination of

low concentration of exogenous auxin and BDPUs, 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 localisation 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; Mattson *et al.*, 2003; Aloni *et al.*, 2003; Nakamura *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 visualisation 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 in cells more efficiently or because of different metabolism. At the same time, it is clear that in the presence of the same type and concentration of auxin, the supplementation of each 5-BDPU concentration causes a magnification of the *DR5::GUS* expression, 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, 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 a gene induced in the presence of exogenous auxin in *Pinus* cuttings during adventitious rooting

BDPUs influence auxin localisation at specific sites during adventitious root formation at the base of pine cuttings, specifically at 6 days of the root initiation process (when auxin-induced cell divisions occurred in the vascular system). The accumulation of IAA at

characteristic areas around the resin canals could due to the capacity of 5-BDPU 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 and 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 rooting response obtained in the presence of higher auxin concentrations, especially when a stronger auxin, such as NAA, is used. The effect of BDPUs on the expression of two genes (*PrSCL1* and *PrSHR*) induced during the early stages of adventitious rooting (24 h after the root induction) (Sánchez *et al.*, 2007; Solé *et al.*, 2008), shows that BDPUs induce the auxin-responsive *PrSCL1* expression in the presence of low auxin concentration (**Fig. 16A**). Otherwise, BDPUs did not affect the expression of *PrSHR* (**Fig. 16B**), a gene that does not respond to exogenous auxin in this system (Solé *et al.*, 2008). This result could indicate that the effect of BDPUs could be executed via the auxin-signalling pathway, directly or indirectly, before the activation of cell divisions and before detectable changes of auxin distribution that originate the root primordia.

Interaction among BDPUs and cytokinin signalling pathway

While auxins promote the adventitious root formation, the inhibitory effect of cytokinins on rooting has been well documented. In fact, cytokinins synthesized in the root apex and transported throughout the whole plant are endogenous inhibitors of root formation in the intact shoot. Removal of the root system interrupts the flow of cytokinins to the shoot stimulating the adventitious root formation. Nonetheless, small amounts of cytokinins can be required for good root formation *in vitro*, even in the presence of auxin. As reported by de Klerk *et al.* (2001), adventitious rooting can be enhanced by weak adenine-type cytokinins, possibly because cytokinins can initiate the cell division essential during the initial step of adventitious root formation. Cytokinins are perceived through a

circuit mediated by distinct functions of receptor histidine kinases (HKs), phosphotransfer proteins (HPs) and response regulators (RRs). The cytokinin phosphorelay is unique in that HK proteins require cytokinin for their activation, in that HP proteins act as cytoplasm and nuclear shuttles and in that enabling RR proteins with DNA-binding domains is the nuclear consequence. Three cytokinin receptors, CRE1/AHK4, AHK2 and AHK3 have been described in *Arabidopsis* (Inoue *et al.*, 2001; Suzuki *et al.*, 2001; Yamada *et al.*, 2001). Even if they show different ligand specificity, CRE1/AHK4 provokes a several fold higher response than AHK3 in either *in planta* assay system or *in vitro* bacterial assay (Hwang and Sheen, 2001; Spíchal *et al.*, 2004). In addition, the functional disturbance of *AHK4* in *wol-3 Arabidopsis* mutant affects the function of the other cytokinin receptors (Kuroha *et al.*, 2006). Based on the analysis of *wol-3* mutant, which shows retardation of the primary root growth, no production of lateral roots and enhanced formation of adventitious roots, Kuroha *et al.* (2006) suggested that cytokinin receptors are necessary for the formation of auxin-transport vascular tissues in the hypocotyl, but not in adventitious roots. Here we describe our findings regarding the study of the interaction among BDPUs and cytokinin signalling pathway at the level of AHK4/CRE1 cytokinin-receptor and the cytokinin-inducible *ARR5* response regulator.

BDPUs interact with the *E.coli*-expressed *Arabidopsis* cytokinin receptor CRE1/AHK4 in the presence of cytokinin

The effect of BDPUs on the sensitivity of CRE1/AHK4 was performed by treatments using different BDPU concentrations alone or in combination with different exogenous cytokinins, such as 2iP, BAP and TDZ, in transformed *E. coli* strain expressing the cytokinin receptor CRE1/AHK4. BDPUs are not able to activate the cytokinin signalling pathway *via* the receptor CRE1/AHK4 in the specific *in vitro* bacterial assay, at all the concentration tested (**Fig. 18**), confirming that, in spite of their chemical structure could resemble that of urea-type cytokinin, their adventitious rooting adjuvant activity does not correlate even with a very weak cytokinin-like activity, not detectable in any cytokinin-like assay that has been previously carried out (Ricci *et al.*, 2006). Ricci *et al.* (2006) hypothesized that the adjuvant rooting activity of BDPUs could possibly depend on the ability of these compounds to reduce the endogenous cytokinin activity and act like 'anticytokinins'. Recently, it has been reported that some synthetic BAP-derivatives, which showed

cytokinin antagonist activity, competitively inhibited the binding of natural cytokinin to the *Arabidopsis* cytokinin receptors AHK4 and AHK3 (Spíchal *et al.*, 2008; Nisler *et al.*, 2010). In an attempt to verify whether BPDUs could antagonize the activity of the plant hormone cytokinin at the receptor level, we examined the interaction of BDPUs and cytokinins with AHK4. Surprisingly, the cytokinin-dependent activation of CRE1/AHK4 in the simultaneous presence of BDPUs and cytokinin was several fold greater than that obtained in the presence of the same cytokinin type and concentration alone (**Fig. 18**), indicating that BDPUs did not act as 'anticytokinins' in this bioassay. On the contrary, BDPUs magnify somehow the sensitivity of CRE1/AHK4 to cytokinins. Accordingly to Spíchal *et al.* (2004), we observed that the capacity of different types of cytokinin alone to activate the receptor AHK4 followed the order: 2iP > TDZ > BAP. Similarly, the observed activation of the receptor by the combinations of BDPUs plus cytokinin reflected the aforementioned order. Furthermore, the activity of these mixtures seems independent from BPDU concentration, since similar CRE1/AHK4 activation was observed at all the BDPU concentration tested.

BDPUs modify cytokinin responsiveness in the presence of exogenous cytokinin in P_{ARR5}:*GUS* transgenic plants

The *Arabidopsis* genome encodes >30 ARRs or ARR-like proteins. Whereas some B-type ARRs are capable of directly activating cytokinin-responsive genes, A-type ARRs may serve as repressors to down-regulate cytokinin responses. Some A-type *ARRs* are primary response genes that are rapidly induced by cytokinin. *ARR5* is one of the response regulator genes of the type A two-component system that is rapidly up-regulated by cytokinin within 10 min (D'Agostino *et al.*, 2000). The expression pattern of P_{ARR5}:*GUS* reflects changes in endogenous cytokinin concentration and allows to analyse the tissue-specificity of the cytokinin response *in planta* (D'Agostino *et al.*, 2000: Werner *et al.*, 2003; Aloni *et al.*, 2005; Stolz *et al.*, 2011). The finding that BDPUs simultaneously supplied with cytokinin were highly active in the CRE1/AHK4 assay, prompted us to test whether the most effective mixtures made by 2iP or TDZ plus 5-BDPU were also biologically active in cytokinin-responsive P_{ARR5}:*GUS* transformants of *Arabidopsis*. Accordingly to already reported data (Spíchal *et al.*, 2004), we demonstrate that either 2iP or TDZ are highly active in the *Arabidopsis* reporter gene test (**Fig. 19**). At the same time, it is clear that in

the presence of the same type and concentration of cytokinin, the supplementation of each 5-BDPU concentration causes a magnification of the *P_{ARR5}:GUS* expression, as a higher intensity and extension of the stain is shown. In addition, the combination of 5-BDPU and cytokinin induce a stronger expression pattern in the root tip and the vasculature than in other parts of the plant (**Fig. 19B and D**). Taken together, the results obtained *in planta* by the reporter gene-based assay of *Arabidopsis* confirm the ones obtained by the bacterial assay. We hypothesise that BDPUs simultaneously supplied with cytokinins, somehow affect the cytokinin signalling pathway.

BDPUs and somatic embryogenesis

The production of somatic embryos has extensive practical and commercial applications, particularly for *in vitro* clonal micropropagation, since it allows large scale vegetative propagation of many species. In some cases somatic embryogenesis is favoured over other methods of vegetative propagation because of the possibility to scale up the propagation, to cryopreservation and to genetic transformation. In addition, somatic embryos are used as a model system in embryological studies seeing as they resemble zygotic embryos. Somatic embryogenesis can probably be achieved for all plant species provided that the appropriate explant, culture media and environmental conditions are employed. Auxins, especially 2,4-D, are known to have a crucial role in the induction of somatic embryogenesis in tissue cultures. However, cytokinins alone or simultaneously supplied with auxin can induce somatic embryo formation as well. Here we present the data obtained by analysing the hypothetical somatic embryogenesis adjuvant activity of BDPUs.

BDPUs exhibit somatic embryogenesis adjuvant activity in the presence of exogenous auxin in *Medicago truncatula* line R108

The effect of BDPUs on the embryogenic capacity was performed by treatments using different BDPU concentrations alone, binary mixtures made by BDPUs and auxin or cytokinin, or ternary mixtures made by BDPUs and auxin and cytokinin using leaf explants

of *Medicago truncatula* line R108. Since embryo formation can be achieved following simultaneous exposure to auxin and cytokinin (Hoffmann *et al.*, 1997), this line is suitable to study the adjuvant activity of BDPUs in the somatic embryogenesis process. BDPUs were ineffective when supplemented alone or in combination with BAP to leaf explants (data not shown), suggesting that BDPUs do not show any 'embryogenic activity' *per se* and do not replace auxin in the mixture. Explants cultured in the presence of 2,4-D alone develop callus and sporadically form some embryos (**Fig. 20A and B**), confirming that both auxin and cytokinin are essential to observe the best embryogenic response. The binary mixtures consisting of higher 6-BDPU concentrations plus 2,4-D determined a similar embryogenic response to that observed with binary mixture made by 2,4-D plus BAP (**Fig. 20A and B**), suggesting that 6-BDPU might replace the cytokinin in the mixture. This observation is in agreement with already reported results from *Citrus* (Carra *et al.*, 2006). *Citrus* is one of the few systems in which somatic embryogenesis can be achieved following exposure to cytokinin alone. Carimi *et al.* (1995) reported that somatic embryogenesis from style-derived callus of *Citrus* cultivars was possible by treatment with BAP. When the effect of MPDUs on embryogenic competence of stigma and style of three *Citrus* species was investigated, it turned out that 2,3-MDPU and 3,4-MDPU alone induced higher percentage of *C. madurensis* and *C. limon* embryogenic explants, respectively, than that obtained with the same concentration of BAP and 4-CPPU (Carimi *et al.*, 1995; Carra *et al.*, 2006). In an attempt to verify whether BDPUs might magnify the effect of the cytokinin even in this system, we supplied to leaf explants ternary mixtures made by BDPUs plus auxin plus a tenfold lowered concentration of cytokinin, as to that of the control condition. No significant difference was detected among treatments and controls (auxin plus high or low concentration of BAP) (**Fig. 20D, E and G**), indicating that BDPUs might be able to enhance neither auxin nor cytokinin activity in these mixtures.

BDPUs and shoot regeneration

Shoot organogenesis is the *de novo* development of shoots from non-meristematic tissues *in vitro*. This phenomenon has long been a subject of interest and practical utility because it provides the basis for asexual plant propagation and the genetic engineering of plants. The *in vitro* morphogenic responses of cultured plants are mostly affected by different components of the culture media. Auxins, cytokinins and the auxin/cytokinin ratio are known to influence shoot formation. The hormonal control of this process has been studied mainly by exogenous application of plant growth regulators. The regeneration of shoots usually includes callus induction and formation from an initial explant, and shoot stimulation and development from the induced callus. Here we describe the results obtained by investigating the hypothetical shoot regeneration adjuvant activity of BDPUs.

BDPUs exert shoot regeneration adjuvant activity in the presence of exogenous cytokinin in *Lycopersicon esculentum*

High percentage of *in vitro* shoot regeneration from tomato cotyledon explants can be achieved as a result of exposure to both auxin and cytokinin or to cytokinin alone (Branca *et al.*, 1990; Ricci *et al.*, 2001a; 2001b; 2006). It has already been reported that either BDPUs alone or in combination with the synthetic auxin BOAA was unable to induce shoot regeneration (Ricci *et al.*, 2006). Therefore, the effect of 5-BDPU on shoot regeneration of *Lycopersicon esculentum* cotyledonary explants was verified by treatments with different 5-BDPU concentrations plus different types of cytokinins, such as 2iP, BAP and TDZ. When 5-BDPU was simultaneously supplemented with 2iP, the percentage of explants forming shoots was significantly greater at the highest 5-BDPU concentration than that recorded with 2iP alone (**Fig. 21A**), indicating that 5-BDPU is able to cooperate with 2iP in the tomato cotyledon bioassay.

CONCLUSIONS

The data presented in this PhD thesis demonstrate that:

- The adventitious rooting adjuvant activity shown by BDPUs is conserved in distantly-related herbaceous and woody species.
- BDPUs exert their adventitious rooting adjuvant activity in either the presence or the absence of exogenous auxin, depending on species. When exogenously applied auxin is needed to induce the rooting response in recalcitrant woody species, the treatment with BDPUs allows the use of low auxin concentration to stimulate adventitious root formation. This implies the absence of callus formation at the root emergence site, producing a good quality root system.
- The adventitious rooting adjuvant activity of BDPUs is unrelated to the type of the exogenous auxin which enhances the rooting response.
- The differences in optimal BDPU concentrations are likely the consequence of differences in the strength of the exogenous auxin, IBA and NAA, *i.e.* the stronger the auxin, the lower is the BDPU concentration required to enhance the formation of adventitious roots.
- BDPUs in the simultaneous presence of auxin probably interact with auxin signalling pathway and with auxin distribution before and after the cell division that originates the adventitious root meristem.
- BDPUs in the simultaneous presence of cytokinin can interact with cytokinin signalling pathway. This interaction might favour cytokinin activity.
- BDPUs also exert their adjuvant activity in somatic embryogenesis and shoot regeneration.

All these findings suggest that BDPUs might affect aspecific cellular events at different levels which, depending on nutrient conditions, growth regulator concentrations, type and physiological condition of explants, can give rise to different morphological processes.

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Acknowledgements

The present PhD study was carried out at the Department of Life Sciences of the University of Parma (Italy) and partially at the Department of Plant Biology of the University of Alcalá (Spain). I wish to express my sincere gratitude to all people who have strongly supported my work.

I am grateful to Prof. Ada Ricci for carrying out the adventitious rooting of apple stem slices but above all for her guidance as my supervisor and for challenging me to do my best.

I would like to thank Prof. Carmen Díaz-Sala and Prof. Dolores Abarca for kindly hosting me in their lab and for their sustained support of my work.

I am grateful to Alberto Pizarro Blanco for carrying out the gene expression analysis, for teaching me how to perform the immunolocalisation of IAA in *Pinus* and above all for being such a precious co-worker and friend.

I thank Lucia Dramis for carrying out the histology of *Pinus* and carob tree and Dr. Miriam Gaudenzi for carrying out the adventitious rooting of *Arabidopsis* etiolated seelings.

I am thankful to Dr. T. Mizuno for kindly providing *Escherichia coli* strain expressing CRE1/AHK4, Dr. Sabrina Sabatini for kindly providing *DR5::GUS* seeds, Dr. Pascal Ratet for kindly providing seeds of *Medicago truncatula* line R108 and Dr. Barbara Ruffoni for kindly providing aseptic cultures of carob and strawberry tree.

Many thanks to Dr. Susanna Del Carlo, Stefano and Alessandra (Interdepartmental Biology Library of the Univesity of Parma) for promptly enriching the bibliography of this PhD thesis. Thanks to Dr. Stefano Leonardi for introducing me to the 'R world'. Thanks to Prof. Valeria Rossi for kindly lending 'her' stereomicroscope. A special thank to Andrew Cameron for English editing most of the introduction of this PhD thesis.

Finally,
I am thankful to Dr. Enrico Rolli for acting as co-supervisor and for being such a wonderful colleague and friend.

I am above all grateful to Dr. Michele Carbognani for going with me through this rough and intense journey and for being such a unique life- and lab-mate.

I wish to thank my family (my mum Anna, my dad Giovanni and my sister Barbara) and friends for constantly believing in me and always and everywhere supporting me.