BIOLOGIA PLANTARUM 47 (4): 593-596, 2003/4

BRIEF COMMUNICATION

Thidiazuron-induced high-frequency shoot regeneration from root region of *Robinia pseudoacacia* L. seedlings

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Abstract

High-frequency regeneration of shoots was achieved at root region of seedlings of *Robinia pseudoacacia* L. cultured from seeds on medium supplemented with thidiazuron (TDZ, 1.0 μM). The roots of intact seedlings proliferated and formed a compact callus followed by differentiation of numerous shoots. Corresponding cultures on benzylaminopurine-containing medium exhibited much weaker response. Hypocotyl segments also formed shoots at a lower concentration of TDZ (0.1 μM). The shoots formed on TDZ-containing medium were well-developed and readily rooted on hormone-free medium. The obtained plants after acclimation in culture room survived after transfer to soil.

Additional key words: acclimation, black-locust, seed culture.

The black-locust (*Robinia pseudoacacia* L.), a multipurpose nitrogen-fixing member of *Leguminosae*, is one of the most quickly growing trees cultivated throughout the world for its timber, firewood, fodder, honey, soil-conservation and erosion-control. In view of its economic importance and desirability of elite plants, *R. pseudoacacia* has been subjected to extensive as well as intensive studies of *in vitro* regeneration. Many efforts have been made to regenerate this tree from different explants.

Attempts to micropropagate this tree, employing either benzylaminopurine (BAP) or thidiazuron (TDZ), are slow with poor yield (Chalupa 1983, 1987, Barghchi 1987, Davis and Keathley 1987). Shoots regenerated from callus cultures on auxin and cytokinin-containing medium are tree-specific (Han *et al.* 1993) and are to be tested for their fidelity; particularly in view of their lack of rooting response (Enescu and Jucan 1985). Regeneration through somatic embryogenesis, although a preferred mode of regeneration due to the presence of preformed shoot- and root-pole in an embryo, is possible from immature embryos and occurs in a low frequency (Arrillaga *et al.* 1994). Moreover, an immature embryo as an explant involves not only a tedium of isolation but is also constrained in its availability. This communication describes a relatively simple method of high-frequency regeneration of shoots due to TDZ-treatment of seedlings and seedling explants. The present regeneration system was aimed to be rapid, reproducible and prolific in comparison with BAP-induced shoot formation.

Sun-dried seeds of *Robinia pseudoacacia* L. collected from mature fruits in northern Iran, were scarified with 3.6 M HCl for 30 min, washed thoroughly with sterile distilled water (SDW), sterilized in 0.5 % solution of mercuric chloride for 20 min, rinsed repeatedly in SDW and placed on nutrient medium for germination. The mineral formulation N_6 (Chu *et al.* 1978) was solidified with 0.8 % agar (*Difco, Bacto*) and supplemented with 2 % sucrose and BAP (0.1 - 1.0 μM) or TDZ (0.1 - 1.0 μM). The cultures were maintained at 25 ± 2 °C under continuous light (15 μmol m^{-2} s^{-1}) provided by *Philips* fluorescent tubes. The experiments were repeated many times with identical results. At least 30 - 40 cultures were raised for each treatment. The plantlets formed *in vitro* were washed out of agar medium by keeping for 4 h in distilled water and occasional shaking. These plantlets were transferred to pots containing autoclaved *Soilrite* (vermiculite) moistened with half-strength Murashige and Skoog's nutrients and the entire set-up was covered with polythene to provide a near-sterile high-humidity environment. After one week, the polythene was removed and plantlets were transferred to garden soil in pots.

Received 12 August 2002, *accepted* 17 August 2003.

Abbreviations: BAP - benzylaminopurine, IBA - indole-3-butyric acid, NAA - α-naphthaleneacetic acid, TDZ - thidiazuron (N-phenyl-N'-1,2,3-thiadiazol-5-yl urea).

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The scarified seeds cultured on hormone-free medium showed signs of germination within 3 d and formed complete seedlings after 10 d. The first noticeable change, observed on medium supplemented with 1.0 μM TDZ after an initial process of germination, was an arrested growth of seedlings in general and of root-system in particular. In 30 % of these seedlings, internal proliferation of root and formation of a few shoots directly on it (Fig. 1*A*) were observable within one week of seed germination. In due course the frequency of such seedlings increased to more than 50 % of cultures and finally in all cultures (Table 1) the formation of a compact callus was followed by differentiation of numerous shoot-buds. Within one month of culture these shoot-buds elongated to form shoots (Fig. 1*B*). A decline in response to 20 % of the cultures was found at a lower concentration of TDZ (0.5 μM). At 0.1 μM of TDZ the proliferation of root system did not result in either callus or shoot formation.

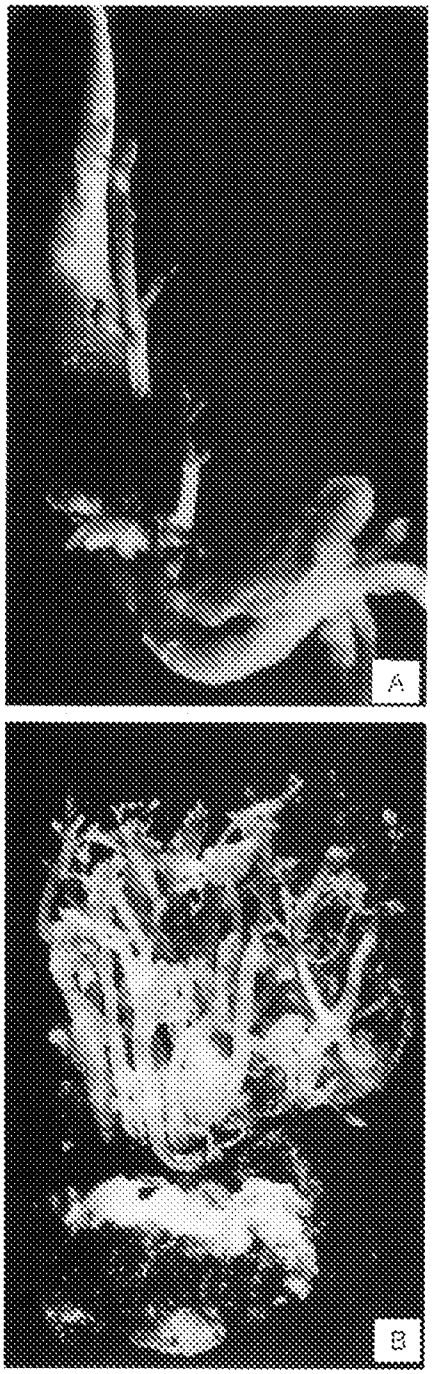

Fig. 1. *Robinia pseudoacacia* seedling on medium with 1.0 μM TDZ. *A* - lower portion of a seedling showing early stage of callusing from root region and differentiation of a couple of shoots; *B* - numerous shoots differentiated on root region of a seedling within 30 d of culture.

Table 1. Effect of TDZ and BAP on regeneration of *Robinia pseudoacacia*.

Explant	Hormone	Conc. [μM]	Response [%]	Number of shoots [culture^{-1}]
Seed	TDZ	1.0	100	54.0 ± 5.25
	TDZ	0.5	20	13.0 ± 2.53
	BAP	1.0	8	2.8 ± 0.23
	BAP	0.5	0	0
Hypocotyl	TDZ	0.1	75	9.1 ± 0.71
	TDZ	1.0	58	5.5 ± 0.45
	BAP	0.1	10	2.0 ± 0.85
	BAP	1.0	36	5.0 ± 0.38

Hypocotyl explants (1 cm) from seedlings developed on hormone-free medium also formed shoots on medium containing TDZ. At a low concentration of TDZ (0.1 μM), formation of calli in about 25 % cultures and direct shoot formation in the remaining cultures (75 %) were observed within 20 d. The shoots originated as a result of *de novo* meristematic activity. On average, nine shoots developed per explant. At a higher concentration of TDZ (1.0 μM) this response declined to 60 % of the cultures with an average of five shoots per explant.

A comparison of these results was performed with corresponding cultures on BAP-containing medium (Table 1). The seedlings developed on medium with 0.1 μM BAP were normal, without any change in morphology. At 1.0 μM BAP, the lower portion of seedlings formed calli, which was followed by formation of 2 - 3 shoot-buds per culture. Hypocotyl segments also produced shoots on medium with BAP. With increasing concentration of BAP from 0.1 to 1.0 μM there was an increase in callusing along with a corresponding enhancement in frequency of cultures showing shoot formation as well as in the number of shoots per explant. At 1.0 μM BAP about 5 shoots were formed per explant.

Shoots developed on roots of intact seedlings or hypocotyl explants in response to TDZ or BAP were well-developed and readily rooted in 70 % cultures on hormone-free medium. Inclusion of NAA (0.5, 1.0 and

2.5 μM) or IBA increased this frequency of rooting to 90 % of cultures. After an acclimation in culture room, these plants survived the transfer to soil.

The regeneration of a few shoots on roots of seedlings of *R. pseudoacacia* in response to BAP and numerous shoots in response to TDZ, reported here represents a new system for rapid regeneration. Utility of seed as an explant allows experimentation all the year round and reduces the optimization of many factors such as size, physiological age and orientation of explant which are crucial for the success of regeneration in general and of legumes in particular (Lazzeri *et al.* 1987, Nef-Campa *et al.* 1996). TDZ-induced shoot formation on roots of seedlings of *Albizzia julibrissin* was reported previously (Sankhla *et al.* 1994, Hosseini-Nasr and Rashid 2002). In addition to shoot formation on root region, the hypocotyl segments of *R. pseudoacacia* also readily formed shoots in response to TDZ. In previous literature shoots formed in response to TDZ were reported to remain stunted (Chalupa 1987, Preece and Imoel 1991, Huetteman and Preece 1993, Sankhla *et al.* 1994). Interestingly this is not the case in black-locust as shown in the present investigation.

The reason for remarkable efficiency of TDZ *vs* BAP in regeneration remains to be resolved. TDZ, a phenylurea derivative exhibits a high morphogenic potential in a diverse array of responses ranging from proliferation of cytokinin-requiring tissue (Mok *et al.* 1987, Thomas and Katterman 1986) to induction of shoot-buds (Thomas and Katterman 1986) and somatic embryos (Hutchinson *et al.* 1996, Iantcheva *et al.* 1999). It promotes shoot regeneration on seedlings of herbaceous as well as woody species (Huetteman and Preece 1993, Liu 1993, Murthy *et al.* 1998). Significantly, it is also effective in regeneration of recalcitrant system such as grain legume (Malik and Saxena 1992). As for mode of action of TDZ at morphological level, it induces an increase in number of meristematic centres on the treated tissue (Beattie and Garrett 1995, Hutchinson *et al.* 1996), also demonstrated in this investigation.

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