Efficient adventitious shoot organogenesis on root explants of *Ocimum basilicum* L.

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Abstract

Adventitious shoot regeneration efficiency of three cultivars of basil (*Ocimum basilicum* L.): 'Grand Vert', 'Fin Vert' and 'Grand Vert Sweet' was investigated in order to develop a mutagenesis and transformation system. Isolated roots from in vitro seedlings were cut into explants of 10-15 mm and cultured in petri dishes containing 25 mL Murashige and Skoog medium (MS) supplemented with sucrose (30 g) and agar-agar (7 g) at pH 5,8. Two different cytokinins were tested individually: N6-Benzyladenine (BA: 0, 1, 5 or 10 µM) and thidiazuron (TDZ: 0, 1, 5 or 10 µM). The maximum percentage of explants showing regeneration (100%) was observed with the cultivar 'Grand Vert' on the medium containing 5 or 10 µM TDZ, in contrast to 10 µM BA that only induced 30% regeneration. Each root explant produced one or more clusters of green nodule-like shoot meristems. When the explants were transferred to MS medium without plant growth regulators, the shoots started to elongate. This is the first report on shoot regeneration on root explants of basil.

Keywords: basil (*Ocimum basilicum* L.), root, shoot organogenesis, 6-benzylaminopurine (BA), thidiazuron (TDZ)

INTRODUCTION

Ocimum basilicum L. (*Lamiaceae*) commonly known as sweet basil is an important culinary herbaceous plant species with aromatic essential oil with antibacterial, insecticidal and fungicidal properties (Oxenham et al., 2005). The oil is also used for pharmaceutical and cosmetic purposes (Lee et al., 2005). The leaves can be used fresh or dried as a spice (Lee et al., 2005). *O. basilicum* has an important economic value. The annual world production of essential oil was estimated at 100 t year^{-1} (Begum et al., 2002). *O. basilicum* is propagated through seeds but the progeny shows variability due to cross pollination (Heywood, 1978). Micropropagation might offer advantages for breeding programs and mass propagation. An adventitious shoot regeneration protocol is also a prerequisite for mutagenesis or genetic modification. Adventitious shoot initiation was reported for different *Ocimum* species, but this was restricted to nodal segment or leaf explants (Banu and Bari, 2007; Shahzad et al., 2012; Sharma et al., 2014; Amutha et al., 2008). The present work describes a protocol for direct organogenesis on root explants.

MATERIAL AND METHODS

Seeds of *O. basilicum* 'Grand Vert', 'Fin Vert' and 'Grand Vert Sweet' were rinsed in 70% ethanol and surface sterilized for 15 min in 10% NaOCl (commercial bleach) with 2 drops of tween in 200 mL of NaOCl and finally rinsed 3 times with sterile distilled water. They were separately placed in test tubes containing 20 mL Murashige and Skoog (1962) macro and micro elements and vitamins (Duchefa), 3% sucrose, 0.6% Plant agar (Duchefa). The pH of the medium was adjusted to 5.8 prior to autoclaving (120°C, 15 min). After 4 weeks, root explants were collected from the in vitro seedlings.

After separation, the roots were cut in small segments (length 10-15 mm) and placed in petri dishes (diameter 90 mm) containing 20 mL MS medium supplemented with 0, 1, 5

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or 10 µM N6-benzyladenine (BA) or thidiazuron (TDZ). The seeds and root explants were incubated at 26±1°C and 16 h photoperiod provided by Greenpower LED Production Module (deep red/white 120 LO, Philips) at 53 µmol m^{-2} s^{-1} PAR. During the 8 h dark period, temperature was 25±1°C. The experiment was repeated 3 times. After 3 weeks, parameters such as number of roots producing callus, secondary roots or clusters of nodule-like shoot meristems were counted for the 3 repetitions. Data were analyzed for significance using ANOVA and the differences were contrasted using Duncan test. All statistical analyses were performed at the 5% level, using the statistical software (SPSS "20,00"). The callus development was accessed with a scale from 0 = no callus, 1 = poor formation (<1 mm^2), 2 = minor formation (1-4 mm^2), 3 = average formation (>4 mm^2). The nodule-like shoot meristems were transferred to hormone free basal medium to elongate and rooting.

RESULTS AND DISCUSSION

In this study, the root explants responded differently depending on cytokinin type, cytokinin concentration and genotype. Explants placed on hormone free media did not respond at all. Small quantities of white callus were induced by 1 and 5 µM TDZ, as well as by 5 µM BA (Figure 1a; Table 1). The formation of nodule-like shoot meristems was observed after 2 weeks of culture. Only 5 and 10 µM TDZ had a significant effect on the number of roots regenerating clusters of nodule-like shoot meristems and on their mean number per explant (Table 1). For 'Grand Vert' 100% of explants produced nodules on the medium containing 5 or 10 µM TDZ, in contrast to 10 µM BA to which only 30% of the roots were respond.

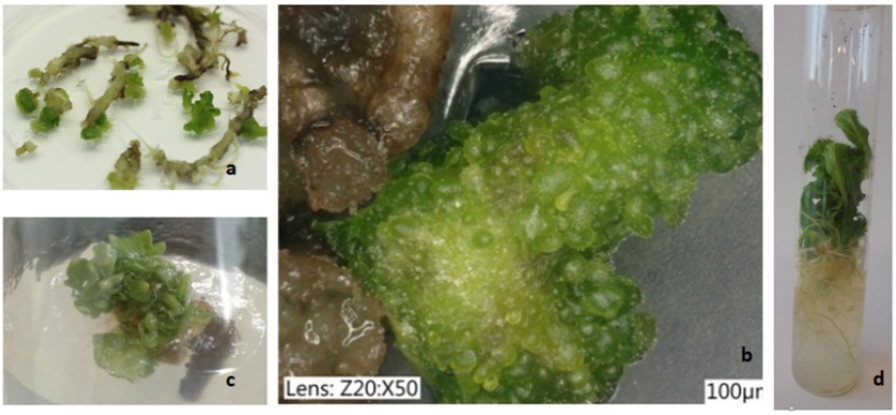

Figure 1. (a) Shoot and callus formation on isolated root, (b) cluster of nodule-like meristems, (c) outgrowth of shoots after transfer of nodule-like meristems in tube, (d) elongated and rooted in vitro shoots.

Table 1. Effects of TDZ and BA on callus formation, secondary roots formation, roots regenerating nodules and number of nodule clusters. Means followed by the same letter are not significant different at 5% level.

		Level of calli formation explant^{-1}			Number of roots explant^{-1}			Roots regenerating nodules (%)			Number of nodule clusters explant^{-1}		
		GV	FV	GVS	GV	FV	GVS	GV	FV	GVS	GV	FV	GVS
TDZ	0	0	0	0	0	0	0	0	0	0	0	0	0
(µM)	1	1	2	1	1	1	1	0	0	0	0	0	0
	5	2	3	2	1	1	1	100a	100a	0	1d	1d	0
	10	3	3	3	1	1	0	100a	100a	100a	1,66a	1,55b	1,33c
BA	0	0	0	0	0	0	0	0	0	0	0	0	0
(µM)	1	0	0	0	0	0	1	0	0	0	0	0	0
	5	0	0	1	0	0	1	0	0	0	0	0	0
	10	0	0	0	0	0	0	30,5b	26 b	7c	1d	1d	1d

Comparison of the different hormones treatments for regeneration revealed that the medium containing 10µM, TDZ gave the best regeneration for the 3 cultivars, compared with BA which gave a relative low percentage of clusters of nodule-like shoot meristems. They were composed of densely packed shoot meristems (Figure 1a) which later formed clusters of shoots on hormone-free medium (Figure 1b). Gopi and Ponmurugan (2006) showed similar structures originating on callus using 2,4-D (2,4-dichlorophenoxyacetic acid) and BA, but called them globular somatic embryos. However, the meristems induced by TDZ do not produce roots and cannot be considered as somatic embryos.

The explant type is known to be an important factor in callus and shoot regeneration efficiency, also in *O. basilicum*. Dode et al. (2003) obtained the highest rate of shoot regeneration from cotyledons of *O. basilicium* using 5 mg L^{-1} BA and 0,2 mg L^{-1} NAA. Using nodal segments, Shahzad et al. (2012) and Szeghi et al. (2014) showed that BA gave the optimum shoot formation frequency.

The large number of meristems present in the clusters of nodule-like shoot meristems (Figure 1c) in our study could be useful for transformation and induced mutagenesis experiments. Those nodules clusters were elongated and rooted in hormone free MS medium (Figure 1d). The maximum number of nodules clusters per explant was 1.66, 1.55 and 1.33, noted respectively on 'Grand Vert', 'Fin Vert' and 'Grand Vert Sweet' with TDZ (Table 1) and were less than 1 per explant with BA. Genotype dependence on adventitious shoot formation in *O. basilicum* has already been shown by several authors (Pattnaik and Chand, 1996; Singh and Sehgal, 1999) and is common in tissue culture (George et al., 2008).

It was noted that TDZ alone produced more shoots per explant (Ekmekci and Aasim, 2014). There is ever increasing evidence that TDZ induces diverse morphogenic responses. It promotes the growth of callus cultures of *Phaseolus lunatus* (Mok et al., 1982) and induces adventitious shoots on mulberry cotyledons (Thomas, 2003). TDZ has a dual action. It is an inhibitor of Cytokinin Oxidase/dehydrogense (Chatfield and Armstrong, 1986) and it strongly activates the cytokinin receptors AHK3 and AHK4 (Spíchal et al., 2004). This dual action might explain its very strong activity in adventitious shoot formation in many dicotyledonous plant species. TDZ has also been suggested to modulate endogenous level of auxin (Murthy et al., 1998).

CONCLUSION

In this study, a simple and reliable protocol for shoot regeneration through adventitious shoot organogenesis has been presented. It shows that TDZ has the potential to induce a large number of shoot meristems on root explants, although its effectiveness depends on the genotype. The highest frequency of shoot formation was observed with 10 µM TDZ, 'Grand Vert' being the most responsive genotype. This protocol could be advantageous for micropropagation, mutation breeding and transgenic plant production of sweet basil.

ACKNOWLEDGMENTS

We wish to thank the Tunisian Ministry of Education for the scholarship and the Laboratory for Applied in Vitro Plant Biotechnology, Department of Plant Production, Ghent University, Belgium for their hospitability.

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