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BRIEF COMMUNICATION

In vitro direct organogenesis and regeneration of *Medicago sativa*

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Abstract

A rapid and efficient plant regeneration protocol for a wide range of alfalfa genotypes was developed *via* direct organogenesis. Through a successive excision of the newly developed apical and axillary shoots, a lot of adventitious buds were directly induced from the cotyledonary nodes when hypocotyl of explants were vertically inserted into modified Murashige and Skoog (MS) medium supplemented with 0.025 mg dm^{-3} thidiazuron (TDZ) and 3 mg dm^{-3} $AgNO_3$. When the lower part of shoots excised from explants were immersed into the liquid medium with 1.0 mg dm^{-3} α-naphthaleneacetic acid (NAA) for 2 min, and then transferred to hormone free half-strength MS medium, over 83.3 % of the shoots developed roots, and all plantlets could acclimatize and establish in soil. The protocol has been successfully applied to eight genotypes, with regeneration frequencies ranging from 63.8 to 82.5 %.

Additional key words: alfalfa, cotyledonary node, mature embryo, silver nitrate, thidiazuron, vitrification

Increased quality, disease resistance and productivity have been the main goals for the improvement of alfalfa (*Medicago sativa* L.), but most of the achievements in the past were obtained by traditional breeding methods rather than genetic engineering techniques (Volenec *et al*. 2002). This is because of the lack of successful regeneration protocol for a wide range of alfalfa genotypes. Some progress has been reported *via* indirect organogenesis or somatic embryogenesis on a variety of explants of alfalfa (Zagorska *et al*. 1997, Gilmnur *et al*. 1987, Barbulova *et al*. 2002, Tian *et al*. 2002, Liang *et al*. 2003), but mostly based on a very small number of highly tissue culture-adapted germplasms did not overcome the limitation of genotype-dependence. Furthermore, compared with the direct regeneration protocols, plants regenerated from tissue or organ with a stage of callus formation could increase the possibility of somaclonal variation (Piccioni *et al*. 1997, Loureiro *et al*. 2007). Therefore, the objective of this study was to establish a rapid and efficient plant regeneration protocol for a wide range of alfalfa genotypes *via* direct organogenesis.

Seeds of *Medicago sativa* L. cv. Eureka were surface sterilized in 75 % ethanol for 2 min and in 0.14 % $HgCl_2$, for 15 min, followed by several rinses with sterile water and dipped in a shallow layer of water. After the overnight imbibition at 4 ºC, seed coats, cotyledons, and radicles were removed (Fig. 1*A*). The cotyledonary nodes with 1 - 2 mm segments of hypocotyl were cultured in modified Murashige and Skoog (1962; MS) media (mMS) consisted of MS salts, B5 vitamins (Gamborg *et al.* 1968), 30 g dm^{-3} sucrose, 8 g dm^{-3} agar and 0 - 1.0 mg dm^{-3} thidiazuron (TDZ). After culture for 10 d at temperature of 25 ºC and 14-h photoperiod (fluorescent tubes providing irradiance of 40 μmol m^{-2} s^{-1}), the main shoot developing from cotyledonary node was excised and explants were subcultured onto mMS media with different TDZ concentrations (0.01 - 0.1 mg dm^{-3}). Six days later, the newly developed axillary shoots were removed and the explants were subcultured onto various media using different methods: *1*) the explants were placed horizontally into mMS medium contained 0.05 mg dm^{-3} TDZ with or without 3.0 mg dm^{-3} $AgNO_3$; *2*) the hypocotyls were inserted vertically into mMS media with different TDZ concentrations (0.01 - 0.05 mg dm^{-3}) and $AgNO_3$ (0 or 3.0 mg dm^{-3}). $AgNO_3$ was filter sterilized and then added to these autoclaved media. The percentage of explants with normal or vitreous shoots as well as the number of normal shoots per explant were recorded after twice 14-d subcultures. To test the influence of genotype on plant regeneration, the optimal adventitious shoot induction

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Abbreviations: MS - Murashige and Skoog; NAA - α-naphthaleneacetic acid; TDZ - thidiazuron.

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procedure was applied to other seven cultivars of alfalfa (Eureka, WL-414, Empress, Derby, Sitel, Sanditi, Powerplant, and Pondus). Each treatment was replicated 3 times, and each replicate consisted of 68 - 75 explants.

Four weeks later, shoots more than 2 cm in height were excised and transferred to various media to induce rooting. One procedure was direct inserting the excised shoots into the half-strength MS (1/2MS) media with different NAA concentrations (0 - 1.0 mg dm^{-3}). An alternative procedure was immersing the lower part of shoot, which was scraped several times by scalpel, into the liquid medium containing 1.0 mg dm^{-3} NAA for 2 min, then transferred to hormone free 1/2MS medium. After three weeks, the percentage of shoot producing a root system was determined. Each treatment was replicated three times, and each replicate consisted of 24 shoots. All data were analyzed using analysis of variance (*ANOVA*) and significance was determined at $P < 0.05$ level. Following root formation, the morphologically normal plantlets were trasferred to sterile mix of soil, peat and *Perlite* (1:1:1) and grown in greenhouse.

For histological examinations, axillary shoots cultured on mMS medium containing 0.05 mg dm^{-3} TDZ and 3.0 mg dm^{-3} $AgNO_3$ for 5 d after excision, were fixed in formalin : glacial acetic acid : ethanol (5:5:90, v/v/v) for 24 h, dehydrated with a graded series of alcohol, and then embedded by paraffin wax. Embedded tissues were cut into 4 µm thick longitudinal sections with a rotatory microtome. Sections were stained with hematoxylin and observed under a light microscope.

Most of explants enlarged in size and only one apical shoot developed from the cotyledonary nodes within 10 d (Fig 1*B*). Although the apical shoot of explants could develop on all media with various TDZ concentrations (0 - 1.0 mg dm^{-3}) (data not shown), the best induction (91.7 %) and development of apical shoot was obtained on mMS medium containing 0.1 mg dm^{-3} TDZ. At the tenth day, newly developed apical shoot was removed and explants were subcultured onto the same media. However, only two axillary shoots developed from no more than 55.2 % of explants (Fig 1*C*), and from the fourth day most of explants began to form some visible callus. Reducing the concentration of TDZ in the media, greatly increased the percentage of axillary shoot induction (approximately 62.3 % for 0.05 mg dm^{-3} and 84.8 % for 0.01 mg dm^{-3}) and avoided the formation of callus during the whole stage. Recent research on the propagation of *Holarrhena antidysenterica* (Mallikarjuna *et al*. 2007) also showed that TDZ was more effective at lower concentrations.

For the induction of adventitious shoots, at the sixth day, two axillary shoots were also removed, and explants were subcultured onto the same media but with 0.05 mg dm^{-3} TDZ. Five days later, many adventitious shoot buds were induced from previous axillary meristems of explants (Fig 1*D*). Similar to our results, Kalia *et al*. (2007) reported that removal of the elongated axillary shoots at regular intervals was necessary to allow the elongation of smaller buds. It suggested that the foregoing failure on the induction of adventitious buds may be partially attributed to the growth of these axillary shoots, which could inhibit the formation of adventitious buds by plant apical dominance. With the growth of adventitious buds,

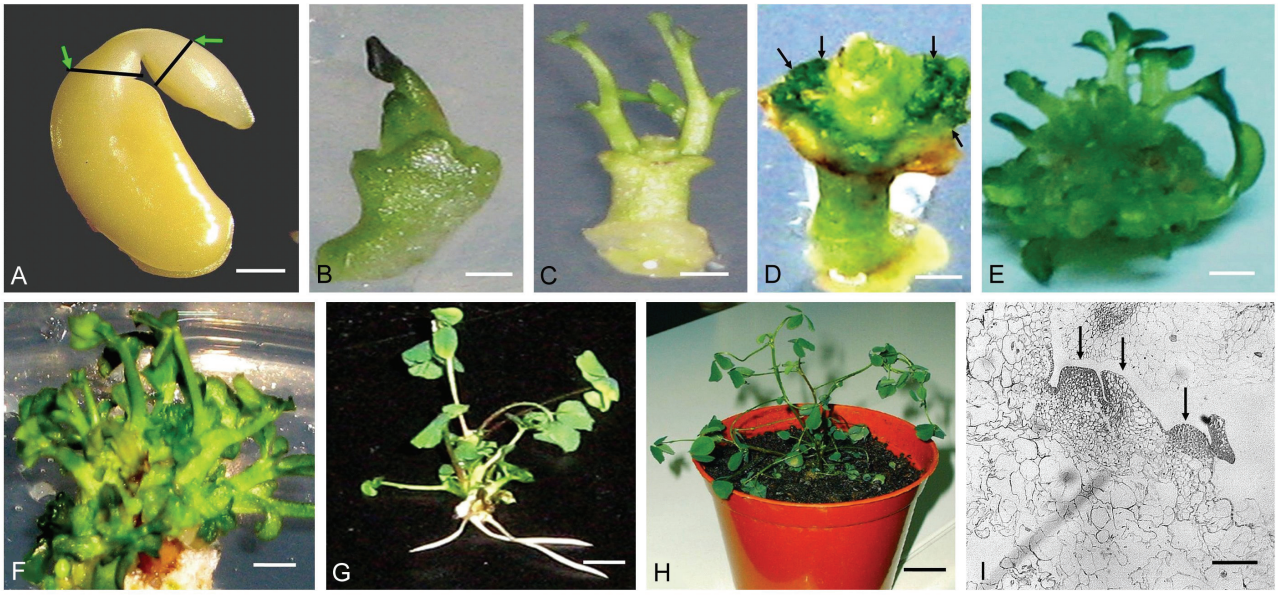

Fig. 1. Different stages of regeneration for alfalfa and histological section of cotyledonary node: *A* - explant was excised (along with the black line) from the "naked" mature embryos of alfalfa (*bar* = 1.0 mm); *B* - explant with one developing apical shoot (*bar* = 2.0 mm); *C* - explant with two developing axillary shoots (*bar* = 2.5 mm); *D* - cluster of adventitious buds (*black arrow*) were induced from the cotyledonary nodes (*bar* = 3.0 mm); *E* - explant with vitreous adventitious shoots (*bar* = 4.0 mm); *F* - growth of morphologically normal adventitious shoots (*bar* = 4.0 mm); *G* - rooted shoots (*bar* = 1.0 cm); *H* - acclimatized plant (*bar* = 7.0 cm); *I* - longitudinal section of explants showing a cluster of new meristems (*bar* = 200 µm).

Table 1. Influence of TDZ, $AgNO_3$ and culture methods on induction of adventitious shoots and their vitrification. The percentage of explants with normal or vitreous shoots as well as the number of normal shoots per explant were recorded after two 14-d subcultures. Values followed by different superscript indicate significant differences ($P < 0.05$), and all values represent means ± SD. NR - data were not recorded due to the serious vitrification.

Culture method	TDZ [mg dm^{-3}]	$AgNO_3$ [mg dm^{-3}]	Explants with adventitious shoots [%]	Explants with vitreous shoots [%]	Number of shoots [$explant^{-1}$]
Horizontal	0.05		97.2 ± 1.3^a	94.4 ± 1.7^a	NR
Horizontal	0.05	3.0	95.9 ± 2.7^a	93.0 ± 2.7^a	NR
Vertical	0.05		87.5 ± 1.2^c	57.2 ± 2.0^b	NR
Vertical	0.05	3.0	97.2 ± 1.4^a	57.0 ± 3.5^b	NR
Vertical	0.025		92.4 ± 1.0^b	12.8 ± 2.9^c	3.8 ± 0.4^b
Vertical	0.025	3.0	97.2 ± 1.4^a	16.3 ± 2.8^c	5.9 ± 0.2^a
Vertical	0.01		89.5 ± 2.9^b	11.5 ± 2.9^c	2.8 ± 0.7^c
Vertical	0.01	3.0	95.9 ± 2.7^a	12.3 ± 5.3^c	4.2 ± 0.2^b

unfortunately, most of them turned into translucent stunted shoots or thickened, turgid and brittle leaves with a glassy appearance (Fig 1*E*), which was called "vitrification" in plant. This phenomenon has also been observed in some previous studies (Zhou *et al*. 2000, Ozden-Tokatli *et al*. 2005, Siddique and Anis 2007).

Li *et al*. (2003) proposed that excess of cytokinins along with the high water potential of the medium were the major reasons for the vitrification of plantlets. In our experiments, therefore, an alternative method (inserting the hypocotyl of explants vertically into the media instead of transversely putting them on the media) was employed, since it could avoid the direct contact of the newly developed adventitious buds with the solid medium, thereby alleviated the negative influence of water potential on the growth of shoots. The results showed that both changing the culture methods and reducing TDZ concentrations from 0.05 to 0.025 mg dm^{-3}, could significantly reduce the percentage of vitreous shoots (Table 1, Fig. 1*F*). In contrast, Kataeva *et al.* (1991) proposed that excess of cytokinin could act as signals inducing an excessive ethylene production, which through a definite sequence of biochemical reactions, leads to vitrification. However, we found out that appending 3.0 mg dm^{-3} $AgNO_3$, a potent inhibitor of ethylene action (Chong *et al.* 1997), into the medium at the same TDZ level had no positive effects on the alleviation of vitrification (Table 1), which was different from the results reported by Zhou *et al*. (2000). Nevertheless, our results indicated that the addition of $AgNO_3$ was obviously beneficial to adventitious shoots formation (Table 1), which was consistent with the results reported by Ozden-Tokatli *et al.* (2005).

Histological observation demonstrated that newly formed adventitious buds were developing entirely from several epidermal cell layers of explants, without intervening a callus stage (Fig. 1*I*). Similar origin of shoot buds has also been reported in other studies (Derek *et al*. 1994, Vasudevan *et al*. 2007).

Root initiation could took place on all 1/2 MS media containing different NAA concentrations (0 - 1.0 mg dm^{-3}), but with a low frequency not more than 55.6 % (data not shown). The highest frequency of root induction (83.3 %) was achieved by dipping the lower part of shoots in 1.0 mg dm^{-3} NAA solution for 2 min before transferring them to hormone free 1/2 MS medium. Moreover, compared with the roots induced on the media containing NAA, roots induced from the shoots treated by the later approach developed more rapidly and without the formation of callus at the base of shoot (Fig. 1*G*). Similar high frequencies of root induction were also reported for other plant species such as *Beta vulgaris* (Gürel *et al*, 1995) and *Juniperus phoenicea* (Loureiro *et al*. 2007). Rooted plantlets were transferred to pots containing sterile soil, peat and *Perlite*, and all regenerated plantlets were successfully acclimatized (Fig. 1*H*).

This regeneration system was also successfully applied to other cultivars (WL-414, Empress, Derby, Sitel, Sanditi, Powerplant, and Pondus) of alfalfa, plant regeneration frequencies, expressed as percentage of explants producing adventitious shoots, ranged from 63.8 - 82.5 % for all tested cultivars. The system developed in this study offers new possibilities for micropropagation and genetic manipulation of alfalfa due to the high efficiency of this protocol for plant regeneration.

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