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Effect of Plant Growth Regulators on Plant Regeneration Through Somatic Embryogenesis of *Medicago sativa* L.

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Abstract

An efficient plant regeneration system in alfalfa (*Medicago sativa* L.) through somatic embryogenesis was established. Embryogenic callus was obtained by culture of hypocotyl segments on MS medium with 0.02 mg L^{-1} IAA and 1.0 mg L^{-1} zeatin after 45 days of culture. Embryogenic calli were converted to the somatic embryos when transferred to either MS medium without plant growth regulators (PGRs) or MS medium containing various cytokinin (BA, kinetin and zeatin). Most of the somatic embryos were developed into plantlets on MS medium supplemented with 0.1 mg L^{-1} kinetin. Also, secondary embryos appeared on the surface of primary embryo but they showed abnormal growth. Regenerated plantlets were transplanted to pots containing vermiculite and perlite for further anaylsis.

Key words: Alfalfa, plant growth regulators, regeneration, somatic embryogenesis

Introduction

Alfalfa (*Medicago sativa* L.) is one of the most important leguminous forage plants cultivated world-wide and is an important perennial forage crop for dairy and beef cattle, poultry and other livestock. Also, alfalfa is often considered as the ideal plant to use in the large-scale production of transgenic products and as functional vegetable because it contains abundant vitamin A, E and protein. Considerable progress has been made to exploit genetic engineering to improve alfalfa. To develop transgenic plants containing useful genes, an efficient plant regeneration system has to be established in *M. sativa*. Till now, there are a few reports on tissue culture and plant regeneration through somatic embryogenesis in *M. sativa* (Pluhar et al. 2001). Somatic embryogenesis is a good developmental model for zygotic embryogenesis and is highly amenable to experimentation. This study was carried out to examine the effects of plant growth regulators on plant regeneration through somatic embryogenesis.

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Materials and Methods

Seeds of alfalfa were surface sterilized in 2% (V/V) NaOCl using commercial bleach for 30 min followed by three washes with sterile distilled water. They were then germinated in the dark at 25±1℃ for 1 week on half-strength MS medium (Murashige and Skoog, 1962) with 3% sucrose and 0.8% agar. The pH was adjusted to 5.8 before adding agar and then autoclaved at 121℃ for 15 min. The cultures were incubated at 25±1℃ and in 16-h photoperiod.

Three investigations were conducted. Firstly, the effect of plant growth regulators (PGRs) on embryogenic callus induction was tested using MS medium containing various PGRs (NAA, 2, 4-D, IAA, BA, kinetin and zeatin). Secondly, the response of explants to embryogenic callus induction was examined using hypocotyl and cotyledon segments of alfalfa seedlings germinated *in vitro*. Hypocotyl and cotyledon segments from 7-day-old seedlings (approximately 7-mm-long hypocotyl) were placed on the surface of solid MS medium supplemented with 2,4-D/kinetin, NAA/BA, and IAA/zeatin combination. Thirdly, the effect of cytokinin on the plant regeneration from embryogenic callus was examined. The cytokinins included BA, kinetin and zeatin.

The embryogenic calli derived from explants were transferred to MS medium supplemented with BA, kinetin or zeatin using concentrations of 0.01, 0.1 or 0.5 mg L^{-1}, respectively.

After 45 days of culture, somatic embryos induced on the above medium were counted and number of plantlet produced was recorded. In addition, secondary embryos formed on the surface of the primary somatic embryo were investigated. Somatic embryos were transferred to MS medium without PGRs for plantlet regeneration.

Results and Discussion

Effect of plant growth regulators on induction of embryogenic callus

The explants (hypocotyls and cotyledons) from 7-day-old alfalfa seedlings were cultured on MS medium with various PGRs to investigate the effect of the latter PGR on embryogenic callus induction. The results obtained are presented in Figures 1 and 2. After 14 days of culture, the cut end of cotyledon explants became swollen and generally developed calli on the media with different concentrations of PGRs. Most of the embryogenic calli were induced at the cut end of explant. Between 30 - 45 days of culture, some shoots were formed on the surface of compact callus and the cut ends of hypocotyl explant. In most media containing 2,4-D and kinetin, and NAA in combination with BA, callus induction was low and callus growth was poor. But in media supplemented with IAA and zeatin, embryogenic calli were induced, which showed vigorous growth (Figure 2A). Although some shoot-like structures were seen on the media containing 2,4-D/kinetin or NAA/BA, no shoots were formed. In the media containing IAA and zeatin, 1 to 4 shoots were formed. Especially, embryogenic calli and shoots were effectively induced on medium with 0.02 mg L^{-1} IAA and 1.0 mg L^{-1} zeatin. These results suggest that IAA in combination with zeatin is more effective than 2,4-D /kinetin or NAA/BA combinations for inducing embryogenic calli.

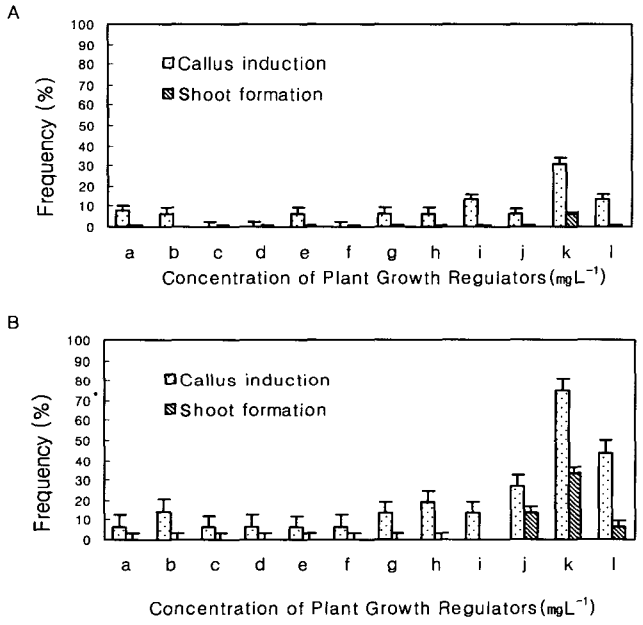

Figure 1. Effect of plant growth regulators on callus induction and shoot regeneration of alfalfa after 45 days of culture
A: cotyledon culture: B: hypocotyl culture
a: control; b: 2,4-D 0.5; c: 2,4-D 0.1+ kinetin 0.5; d: 2,4-D 0.1 + kinetin 1.0; e: 2,4-D 0.5 + kinetin 1.0; f: NAA 0.5; g: NAA 0.1 + BA 1.0; h: NAA 0.1 + BA 0.5; i: NAA 0.5 + BA 1.0; j: IAA 0.01 + zeatin 0.5; k: IAA 0.02 + zeatin 1.0; l: IAA 0.2 + zeatin 2.0 mg L^{-1}.

The effect of various PGRs on somatic embryogenesis has earlier been reported. For instance, 2,4-D can improve the rate of embryogenic cell mass formation but inhibits their further development in mature embryo in *Acanthopanax* (Gui et al. 1991). Compact, friable and embryogenic calli were initiated from young leaves of *Paspalum scrobiculatum* cultured on MS medium supplemented with 2,4-D (Nayak and Sen, 1989). Also, in case of *Brassica nigra*, supplementation with GA_3 enhanced embryogenic response ten-fold (Gupta et al. 1990). In the present study, 2,4-D did not affect the induction of embryogenic callus, Also, no embryogenic callus was formed when explants were cultured on MS medium supplemented with combination of 2,4-D and kinetin or NAA and BA. Most embryogenic calli were induced on medium containing IAA and zeatin. Especially, embryogenic calli were effectively induced on medium with 0.02 mg L^{-1} IAA and 1.0 mg L^{-1} zeatin.

Response of hypocotyl and cotyledon segments on induction of embryogenic callus

In order to compare the response of explants to the embryogenic callus induction, cotyledons and hypocotyls of seedlings were cultured on the same media containing various PGRs. When hypocotyls were cultured on MS medium with various PGRs, the explants showed swelling at the surface within 15 days of culture and the embryogenic callus first appeared as tiny protrusion when observed under a microscope. Such protuberances were concentrated at the cut ends of hypocotyl explants. After 30 days of culture, on MS medium with 2,4-D/kinetin or NAA/BA combinations, hypocotyl segments turned brown and finally died. But, on medium containing 0.02 mg L^{-1} IAA and 1.0 mg L^{-1} zeatin, hypocotyl segments formed tiny yellowsh embryogenic calli and shoots were formed at cut ends of swollen hypocotyls

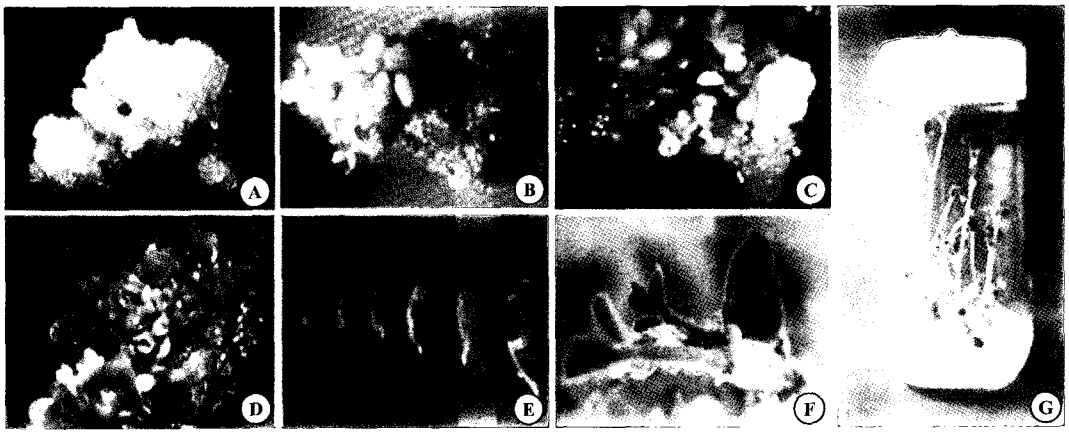

Figure 2. Plant regeneration through somatic embryogenesis in tissue culture of alfalfa (*M. sativa*). A: Embryogenic callus (EC) formation on MS medium with 0.01 mg L^{-1} IAA and 1.0 mg L^{-1} zeatin; B: Somatic embryos of various shape produced from EC; C: Secondary embryos (SE) formed on the surface of primary somatic embryo; D: Multiple SE with fused cotyledons; E: Developmental stage of somatic embryo; F: Direct shoot formed on edge of explant; G: Normal plantlet prior to acclimatization in pot.

(Figure 2F).

Callus induction frequency of 2, 4-D in combination with kinetin, or NAA in combination with BA was less than 20%. But when hypocotyl segements were cultured on medium with IAA and zeatin, induction of embryogenic calli was about 70% and the maximum number of shoots induced from callus was 4 (Figure 1B). When cotyledon explants were cultured on the same media (Figure 1A), the results were same as those for hypocotyl culture. But, hypocotyl explants were better embryogenic callus than cotyledon explants for induction. There are many reports on plant regeneration through somatic embryogenesis using various organs of plant. Immature inflorescences explants of *Setaria italica* showed a higher tendency for regenerating into plantlets through somatic embryogenesis (Xu et al. 1984). Plant regeneration from immature embryos of peanut (Peggy, 1989), oak (Chalupa, 1990) and strawberry (Wang et al. 1984) has been accomplished through somatic embryogenesis. Also, high frequency of direct somatic embryogenesis was observed using cotyledon explant of *Bupleurum falcatum* (Lee et al. 1988). Somatic embryogenesis of cotton (John, 1988) and cassava (James and Graham, 1987) was reported using cotyledon explants. Somatic embryogenesis from leaf explants of rose (Wit et al. 1990) and plant regeneration from cultured leaf tissue explants of *M. truncatula* by somatic embryogenesis have been reported. The frequency of somatic embryogenesis was increased when regenerated plants *in vitro* were used as an explant source (Nolan et al. 1989).

In this study, observations were made 45 days of culture by counting the number of shoots / explant under microscope. Combined over all the culture media, embryogenic callus induction was as more than twice higher for hypocotyls than cotyledon explants.

These results, together with the somatic embryogenesis suggest that hypocotyl explants are more useful for somatic embryogenesis than cotyledon explants in *M. sativa*.

Effect of cytokinin on plant regeneration from emhryogenic callus

Different cytokinins were used to compare their effectiveness for embryogenesis from embryogenic callus (Table 1). The cytokinins included BA, kinetin and zeatin with different concentration (0.01, 0.5 or 1.0 mg L^{-1}). Within 30 days of culture in the media containing all cytokinins, embryos were induced from embryogenic calli (Figure 2B) and developed into plantlets with shoots and roots. When the embryogenic calli were transferred to MS basal medium with 0.1 mg L^{-1} kinetin, 15 shoots were induced, of which 45% formed normal plantlets with roots and leaves (Figure 2G). However, the addition of 0.1 mg L^{-1} zeatin in the medium, most of the calli formed embryos, onto which secandary embryos with abnarmal growth (Figure 2C). Also, multiple secondary embryos were generated from somatic embryo. Most of the secondary embryos derived from the primary somatic embryos were difficult to separate because the cotyledons were fused with embryos (Figure 2D). It has long been observed that appropriate medium can promote embryogenesis (Arnold et al. 2002). In general, somatic embryo induction usually occurs on medium with a high auxin concentration, and the medium without auxin or with low auxin concentration allows embryo development (Ammirato 1983). In *Acanthopanax*, secondary somatic embryos developed when somatic embryos were transferred to medium supplemented with IAA (1-3 mg L^{-1}) or zeatin

Table 1. Effect of cytokinin on somatic embryogenesis and shoot regeneration from calli of alfalfa (*Medicago sativa*) after 45 days of culture[a].

Plant Growth Regulators (mg L^{-1})			No. of explanted callus	No. of explant with SE[b]	No. of shoots	No. of secondary embryo[c]
BA	Kinetin	Zeatin				
Control			20	3	4	9
0.01			20	10	14	11
0.1			19	2	11	17
0.5			20	10	8	19
	0.01		20	2	7	14
	0.1		19	8	15	10
	0.5		18	12	4	16
		0.01	19	13	5	9
		0.1	21	20	13	32
		0.5	20	-	3	-

[a] MS medium was used
[b] Somatic embryo
[c] Numbers contain the multiple secondary embryos generated from a SE

(0.5 mg L^{1}) (Gui et al. 1991). These results suggest that the requisements of PGRs was different for plant species in tissue culture. In the present study, somatic embryos were formed in the medium with cytokinin and regenerated plantlets into (Figure 2E).

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