Plant Biotechnology 23, 431–435 (2006)

Tissue Culture Note

The combined effect of photoperiod, light intensity and GA_3 on adventitious shoot regeneration from cotyledons of spinach (*Spinacia oleracea* L.)

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Received May 8, 2006; accepted June 29, 2006 (Edited by S. Ogita)

Abstract The effects of photoperiod, light intensity and gibberellic acid (GA_3) on adventitious shoot regeneration from spinach cotyledons were tested. The Analysis of Variance F test showed that the effects of photoperiod and GA_3 on shoot regeneration were significant at high light intensity of 90–100 μmol $m^{-2} s^{-1}$. The combined effect of optimum shoot regeneration and highest shoot multiplication was observed in explants of short day (SD)-grown seedlings cultured under the SD condition and at high light intensity, with 0.5 mg l^{-1} GA_3 in Murashige and Skoog medium supplemented with 1 mg l^{-1} 6-benzyladenine and 0.4 mg l^{-1} α-naphthaleneacetic acid. Scanning electron microscopy observation revealed multiple shoots regenerating directly from the basal end of cotyledon preceding callus. The regenerated shoots developed into normal plantlets and grew well upon transfer to pots. Some regenerated shoots flowered *in vitro* under the non-inductive SD condition.

Key words: GA_3, light intensity, photoperiod, regeneration, spinach.

Spinach (*Spinacia oleracea* L.) is a leafy vegetable of the *Chenopodiaceae* family. It is a rich source of vitamins and minerals (Al-Khayri 1995). Spinach is dioecious and flowers during long days. An efficient regeneration system is a prerequisite for the production of genetically modified spinach plants in crop improvement programs for disease resistance and late bolting. Reports on spinach regeneration through somatic embryogenesis and organogenesis have indicated that the type of explant plays an important role: root segments are superior in somatic embryogenesis while cotyledons are efficient in organogenesis (Komai et al. 1996 a,b; Molvig and Rose 1994; Zhang and Zeevaart 1999). Leaf discs, hypocotyls, root segments, leaf-derived protoplasts, thin cell layers from hypocotyls and roots have also been utilized in organogenesis (Al-Khayri et al. 1991, 1992; Goto et al. 1998; Knoll et al. 1997; Leguillon et al. 2003; Mii et al. 1992; Molvig and Rose 1994; Sasaki 1989; Xiao and Branchard 1995; Zhang and Zeevaart 1999). Organogenesis has been utilized for the genetic transformation of spinach by *Agrobacterium tumefaciens* (Al-Khayri 1995; Knoll et al. 1997; Zhang and Zeevaart 1999). Regenerated plants obtained by somatic embryogenesis of explants transformed by root inducing transfer-DNA (Ri T-DNA) of *Agrobacterium rhizogenes* showed abnormal phenotypes (Ishizaki et al. 2002). In spinach regeneration, the role of GA_3 has been highlighted in most occasions in both somatic embryogenesis and organogenesis (Al-Khayri et al. 1992; Komai et al. 1996b; Molvig and Rose 1994; Xiao and Branchard 1993). The effect of exogenous ethylene on embryogenic callus formation in the presence of GA_3 has also been interpreted to facilitate cell response to GA_3 (Ishizaki et al. 2000).

In plant tissue cultures, external factors such as light (wavelength, light intensity and photoperiod) also play an important role, in addition to medium composition (Hughes 1981, Hussey 1986). These factors are not independent but interact in complex ways (Hughes 1981). Responses to photoperiod and light intensity of plant tissue cultures during organogenesis may be species-, variety- and explant-dependant (Economou and

Abbreviations: ANOVA, Analysis of Variance; BA, 6-benzyladenine; IBA, indolebutyric acid; GA_3, gibberellic acid; LD, long day; MS, Murashige and Skoog; NAA, α-naphthaleneacetic acid; SD, short day; SEM, scanning electron microscopy.

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Read 1987). The photoperiod effect associated with GA_3 on spinach life cycle is clear: during long days, spinach tends to bolt and subsequently flower with a morphologic change in rosette plant structure, while exogenous GA_3 supply also simulates this process under the SD condition. Furthermore, gene expression for gibberellin biosynthesis is regulated by such environmental factors as light intensity and photoperiod (Yamaguchi and Kamiya 2000).

The objective of our present experiment is to examine whether photoperiod and light intensity have an effect on the promotive effect of GA_3 on adventitious shoot regeneration *in vitro* because as far as we know, there are no reports of such effects on spinach regeneration.

Cotyledons are good explants as meristem cells are easily formed on their base, which can be directed towards shoot primordium development with the correct hormonal balance (Zhang and Zeevaart 1999). Therefore, we used cotyledons as explants in our experiment. Murashige and Skoog (MS) medium (Murashige and Skoog 1962) (Sigma-Aldrich Co., St. Louis, MI, USA) containing 3% (w/v) sucrose and 0.8% (w/v) agar (Nacalai Tesque Inc., Kyoto, Japan) was used as basal medium in all experiments. The pH of the medium was adjusted to 5.6–5.8 before autoclaving. Twenty five ml of culture medium was poured into a petri dish of 9 cm diameter (BIO-BIK, Ina-Optica Co. Ltd., Osaka, Japan). Seeds of the spinach cultivar Longstanding Bloomsdale Dark Green (Stokes Seeds, Buffalo, NY, USA) were surface-sterilized (Zhang and Zeevaart 1999) and germinated on the basal medium under either the SD condition (8 h light and 16 h darkness) or the long day (LD) condition (16 h light and 8 h darkness). Cotyledons were separated aseptically from 5-d-old seedlings and placed on basal medium containing $1\,mg\,l^{-1}$ 6-benzyladenine (BA) (Wako, Osaka, Japan) and $0.4\,mg\,l^{-1}$ α-naphthaleneacetic acid (NAA) (Wako) as reported by Zhang and Zeevaart (1999), combined with one of four concentrations of GA_3 (Wako) (0, 0.5, 1 or $2.5\,mg\,l^{-1}$), and cultured under either the LD or the SD condition at high light intensity of 90–100 $\mu mol\,m^{-2}\,s^{-1}$ or low light intensity of 5–10 $\mu mol\,m^{-2}\,s^{-1}$. Cultures were kept at 22°C.

Explants were collected after incubation for 4 to 20 days and fixed immediately with FAA (formalin: acetic acid: 70% ethanol, 5 : 5 : 90). The specimens were dehydrated in an ethanol-acetone series, dried at the critical point, coated with platinum, and observed under a scanning electron microscope (SEM) (S-2150; Hitachi Ltd., Tokyo, Japan).

Regenerated shoots 1–3 cm in length were separated from the cotyledons and used for rooting experiments. Reusable plastic jars (60×60×100 cm, Iwaki, Asahi Technoglass Co., Tokyo, Japan) were used with 70 ml of culture medium per jar. Five shoots were placed in one jar and around 25 shoots were used for each treatment. Cultures were kept at high light intensity under the SD condition. Three consecutive experiments were carried out. First, the effect of indolebutyric acid (IBA) (Sigma-Aldrich) concentration (0, 0.025, 0.1, 0.25, 0.5, 0.75 or $1\,mg\,l^{-1}$) in the basal medium was tested because both hormone-free and $1\,mg\,l^{-1}$ IBA MS medium had been used for rooting of shoots formed in GA_3-containing medium (Al-Khayri et al. 1991; Molvig and Rose 1994). Second, a procedure with a pre-treatment was carried out (Zhang and Zeevaart 1999): pre-treatment with $20\,mg\,l^{-1}$ IBA for 2 h was carried out prior to transfer to basal medium containing 0, 0.025 or $0.1\,mg\,l^{-1}$ IBA. Third, shoots were subjected to treatment with two concentrations of IBA (20 and $100\,mg\,l^{-1}$) for two durations (2 h and 5 h) prior to transfer to hormone-free basal medium containing 0.2% activated charcoal (Sigma-Aldrich). Cultures were kept under the SD condition at high light intensity.

The highest shoot regeneration frequency of 47% was observed from SD-grown seedlings cultured at high light intensity with $0.5\,mg\,l^{-1}$ GA_3 (Figure 1). The two-factor factorial Analysis of Variance (ANOVA) F test was carried out to detect differences among the factors tested: the effects of photoperiod and GA_3 concentration on shoot regeneration were significant at high light intensity, whereas no significant effects were observed at low light intensity (Table 1). Fisher's Protected Least Significant Difference (PLSD) multiple range test was used to detect the significant effect of the optimum combination of cotyledons from SD-grown seedlings cultured under SD at high light intensity with $0.5\,mg\,l^{-1}$ GA_3 (Figure 1). SEM was conducted for the early and clear identification of adventitious shoots on the basal end of cotyledons. It was used to get rid of the suspicion of pre-existing axial bud growth near cotyledon base. Several adventitious shoots were observed simultaneously on the abaxial side prior to callus formation (Figure 2A). Each adventitious shoot had an apex and some leaf primordia in a helical arrangement (Figure 2B). Multiple shoots developed normally on the cotyledon base and callus formation continued there (Figure 2C). One to 6 shoots were recorded per shoot-forming cotyledon. The highest shoot regeneration treatment was associated with the highest multiplication frequency of 71%. No significant effects of photoperiod and GA_3 concentration were observed on multiple shoot regeneration. According to our SEM observations, adventitious shoot emergence started as early as one week after culture, showing more rapid regeneration than other reports for cotyledons (Molvig and Rose 1994; Zhang and Zeevaart 1999). Direct multiple shoot regeneration could be a useful feature for the efficient production of independent transformants.

Very high levels of GA_3 were used in previous spinach regeneration protocols (Xiao and Branchard 1995), but

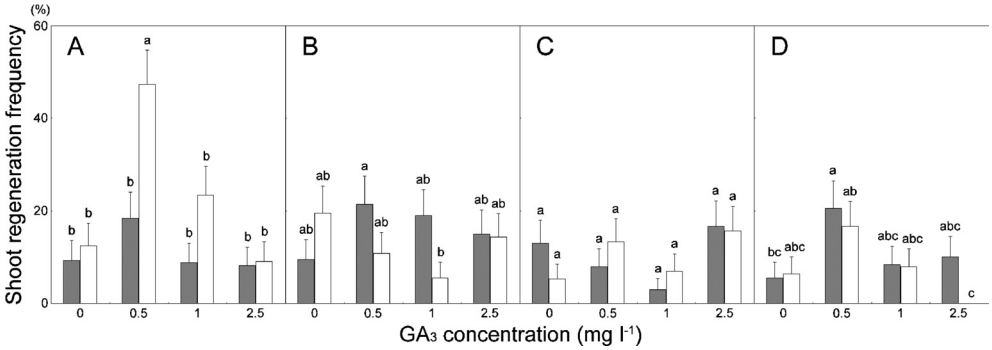

Figure 1. Adventitious shoot regeneration frequency of cotyledon explants. Each bar shows the mean frequency of shoot regeneration in spinach cotyledon explants and error bar shows standard error (n=20–48). Shoot regeneration frequency is expressed as the number of shooted explants per total number of cotyledon explants cultured. The significant differences for all combinations of the two categories were determined using Fisher's PLSD (protected least significant difference) multiple range test at 0.05 confidence level. Values with the same letters in the same figure are not significantly different. Data were recorded after 4 weeks of culture. Gray bars represent low light intensity and white bars represent high light intensity. (A) Cotyledons from SD-grown seedlings cultured under the SD condition; (B) cotyledons from SD-grown seedlings cultured under the LD condition; (C) cotyledons from LD-grown seedlings cultured under the SD condition; and (D) cotyledons from LD-grown seedlings cultured under the LD condition.

Table 1. Effects of photoperiod and GA_3 concentration on shoot regeneration frequency of cotyledon explants cultured at low and high light intensities.

Light intensity	Source of variance	Degrees of freedom	Sum of squares	Mean squares	F-statistic	P-value	
Low	Photoperiod	3	0.39	0.13	1.22	0.3008	
	GA_3 concentration	3	0.63	0.21	1.97	0.1172	
	Interaction effect	9	0.08	0.01	0.08	0.9998	
	Residual	564	60.45	0.11			
	Total	579	61.55				
High	Photoperiod	3	2.17	0.72	6.57	0.0002	*
	GA_3 concentration	3	1.61	0.54	4.86	0.0024	*
	Interaction effect	9	0.83	0.09	0.83	0.5851	
	Residual	579	63.90	0.11			
	Total	594	68.51				

Shoot regeneration frequency of cotyledon explants is expressed as the number of shooted explants per total number of cotyledon explants cultured. Asterisk indicates significant difference in shoot regeneration frequency at $\alpha=0.05$, following two-factor factorial ANOVA F test.

in our present study, clusters of slender shoots were rarely observed or only calluses were observed at the highest GA_3 concentration tested (2.5 mg l^{-1}). GA_3 is known to promote shoot primordium development (Molvig and Rose 1994), but its effect depends on the cultivar (Al-Khayri et al. 1991; Komai et al. 1996a; Goto et al. 1998; Ishizaki et al. 2001), the type of explant (Komai et al. 1996a), the effective concentration (Al-Khayri et al. 1992), the composition and the sequence of plant hormones in the medium (Molvig and Rose 1994) and the photoperiod and light intensity, as shown in our present results.

Root formation frequencies when 0, 0.025, 0.1, 0.25, 0.5, 0.75 or 1 mg l^{-1} IBA basal medium was used ranged from 5% to 17.6% in the IBA experiment without pre-treatment. In contrast, pre-treatment with 20 mg l^{-1} IBA for 2 h was effective for rooting, and the shoots cultured in 0, 0.025 and 0.1 mg l^{-1} IBA after the pre-treatment rooted at frequencies of 10%, 36.0% and 30.7%, respectively. The root formation was observed during the period of 1 to 6 weeks. The average root length exceeded 10 cm after 6 weeks, and the roots were well branched and profuse especially at low concentrations of IBA in the rooting medium with or without the IBA pre-treatment (Figure 2D). In the next experiment, activated charcoal was added to prevent the possible negative effects of residual cytokinin from the shoot formation medium. The root formation frequencies in the case of pre-treatment with 20 mg l^{-1} IBA for 2 h and 5 h durations were 11.7% and 10.5%, respectively, suggesting that activated charcoal has no effect on spinach rooting. By contrast, no root formation was observed in the case of pre-treatment with 100 mg l^{-1} IBA. Therefore, we concluded that pre-treatment with 20 mg l^{-1} IBA for 2 h followed by 0.025 mg l^{-1} IBA in the basal medium is the optimum condition for root formation. Several regenerants produced flowers during the culture period for rooting under the non-inductive SD condition (Figure 2D). *In vitro* flowering of regenerated shoots grown in GA_3-containing medium under both the SD (Al-Khayri 1995) and the LD conditions (Molvig and Rose 1994; Xiao and Branchard 1995; Komai et al.

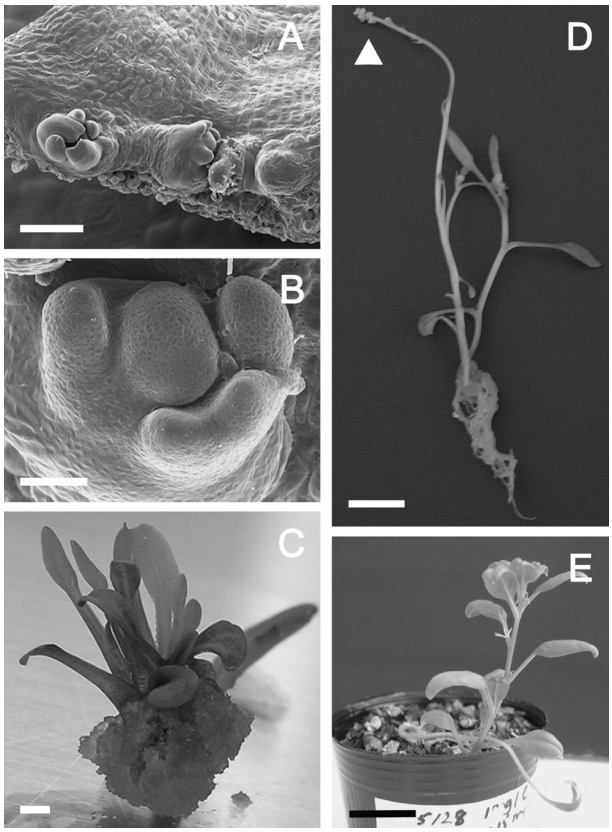

Figure 2. Adventitious shoot regeneration in spinach cotyledon explants from SD-grown seedlings cultured under the SD condition and at high light intensity with 0.5 mg l^{-1} GA_3. (A) Adventitious shoots regenerated at the basal end on the abaxial side of the explant. Bar=0.5 mm. (B) Apical meristem and leaf primordia of an adventitious shoot. Bar=100 μm. (C) Regenerating shoots and callus formed on the basal part of the cotyledon explant after 4 weeks of culture. Bar=1 mm. (D) Root formation from a regenerated shoot after 6 weeks of culture in rooting medium containing 0.025 mg l^{-1} IBA after pre-treatment with 20 mg l^{-1} IBA for 2 h. An incipient male inflorescence is indicated by a white arrowhead. Bar=1 cm. (E) A regenerated plant in a pot after 15 weeks of explant culture. Female flowers are at the leaf axils. Bar=1 cm.

1999) was also reported. *In vitro* flowering can be exploited to shorten the time required for seed production from regenerated transgenic shoots crossed with wild type in transgenic programs. The *in vitro* flowering under the non-inductive SD condition is probably due to the activation of floral integrator(s) not by the photoperiod pathway but by the gibberellin pathway and/or culture stress. The rooted shoots were transferred to pots where they grew vigorously (Figure 2E). In conclusion, our report reveals that high light intensity and the SD condition are effective for rapid regeneration of multiple shoots from spinach cotyledons directly, when combined with the effective GA_3 concentration and the suitable auxin-to-cytokinin balance in the medium.

Acknowledgements

This work was supported by the program for Promotion of Basic Research Activities for Innovative Biosciences (PROBRAIN).

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