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Full Length Research Paper

The effect of plant growth regulators, explants and cultivars on spinach (*Spinacia oleracea* L.) tissue culture

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Spinach (*Spinacia oleracea* L.) is an important vegetable crop of which dioecy in nature has made cultivar improvement difficult using traditional breeding methods; therefore, production of high amount of disease free spinach is critical. To achieve the best explants and media for spinach tissue culture, the effects of two different plant growth regulators, two explants and cultivars on adventitious shoot regeneration were tested. The Analysis of Variance (ANOVA) showed that the effects of plant growth regulators on spinach tissue culture were significant; moreover, the effects of explants were not significant except on the regeneration phase. The best medium for callous induction was MS media containing 1.5 mgl^{-1} IAA + 2.5 mgl^{-1} GA3. The best medium for shoot regeneration was MS media contained 0.5 mgl^{-1} NAA + 2 mgl^{-1} GA3. The best rooting medium was MS medium containing 0.5 mgl^{-1} IBA. Results presented inhibitory effect of GA3 for callus and root formation; whereas, promote shoot development.

Key words: Plant growth regulators, explants, cultivars, tissue culture, spinach.

INTRODUCTION

Spinach (*Spinacia oleracea* L.) is a leafy vegetable of the Chenopodiaceae family (Al-Khayri, 1995). Spinach is dioecious and genetically is a diploid with 2n = 12 chromosomes (Khattak et al., 2006). It requires long days, moderately deep and highly fertile soil for its growth (Akhtar et al., 2008). 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 (Geekiyanage et al., 2006)

In spinach tissue culture, although callogenesis was described by Hildebrandt et al. (1963), Neskovic and Radojevic (1973) reported on the first adventitious bud formation only in 1973. Several authors have described different methods for in vitro multiplication of spinach, via indirect callogenesis (Sasaki, 1989; Al-Khayri et al., 1991; Mii et al., 1992; Xiao and Branchard, 1995) and somatic embryogenesis (Xiao and Branchard, 1993; Komai et al., 1996a). Spinach regeneration through somatic embryogenesis and organogenesis have indicated that the type of explants plays an important role: root segments are superior in somatic embryogenesis while cotyledons are efficient in organogenesis (Komai et al., 1996 a,b;

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Abbreviations: MS, Murashige and Skoog; **IAA,** indole-3-acetic acid; **IBA,** indol butyric acid; **NAA**, α –Naphthalene acetic acid; **GA3**, gibberlic acid; **BAP**, 6-Benzyl-amino purine; **KIN**, 6-Furfuryl amino purine; **CRD**, completely randomized design; **SD**, short day.

Molvig and Rose, 1994; Zhang and Zeevaart, 1999). Cotyledons (Molvig and Rose, 1994; Zhang and Zeevaart, 1999), mature seeds (Al-Khayri et al., 1992), 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)

Successful results were obtained from protoplasts (Goto et al., 1998). A strong genotypic effect is commonly observed in spinach tissue culture and a given treatment is rarely well-suited to several genotypes even within a given research team (Al-Khayri et al., 1991; Mii et al., 1992; Komai et al., 1996a). Kumar and co worker studied the effects of NAA and BAP on spinach shooting and they presented that MS media containing 2 mgl^{-1} NAA was the best media for shooting (Kumar et al., 2001). In addition, Knoll and colleague demonstrated that optimum shoot regeneration was from explants of apical and middle root regions on medium with 20 mM NAA and 5.0 mM GA3 (Knoll et al., 1997).

The role of GA3 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; Geekiyanage et al., 2006). The effect of exogenous ethylene on callus formation in the presence of GA3 has also been interpreted to facilitate cell response to GA3 (Ishizaki et al., 2000). Al-Khayri et al. (1992) showed existing GA3 in the high level play the major role in callogenesis. Geekiyanage et al. (2006) reported that 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 mgl^{-1} GA3 in MS medium supplemented with 1 mgl^{-1} BAP and 0.4 mgl^{-1} NAA. Friable calluses induced from root segments with a high amount of growth regulators 48.52 µM IAA and 10 µM GA3 were reported (Xiao et al., 1997). Some Researchers indicated that the best organ for regeneration is cotyledons (Zhang and Zeevaart, 1999; Geekiyanage et al., 2006), some other represent another organs such as hypocotyl (Kabiri et al., 2006; Sasaki, 1989).

MATERIALS AND METHODS

The objective of our present experiment is to examine the effect of different plants growth regulators and explants on spinach tissue culture because as far as we know, there are no reports of such effects on spinach regeneration. Seeds of the spinach cultivars of Karaj local seedlings and Melody were surface sterilized. The treatments applied consisted of successive immersions in tap water, 70% (v/v) ethanol for 2 min and 5% (w/v) calcium hypochlorite for 30 min. The seeds of both genotypes were finally rinsed three times in sterile distilled water.

For germination, they were placed on moist filter paper in Petr dishes, in short day condition (8 h light and 16 h darkness) at 24 ±1°C. All media were adjusted to pH 5.8, before autoclaving at 120°C for 20 min; IAA, NAA and GA3 were filter-sterilized. For the callous induction studies, explants were excised from cotyledons and hypocotyls of 5-day-old plantlets. They were cultured in 9-cm Petri dishes on Murashige and Skoog; 1962, (MS) medium supplemented with 30 gl-1 sucrose, 8 gl-1 agar and with IAA (0, 1 and 1.5) and GA3 (0, 2 and 2.5) either single or combinations. The callous formation character was evaluated. A completely randomized design was used to conduct the experiments. For each medium tested, there were 4 replicate explants and response was measured. All cultures were maintained at 24 ± 1°C under short day condition (8 h light and 16 h darkness). When calluses grew they were transferred to shoot regeneration medium containing GA3 (0, 2 and 2.5) and NAA (0, 0.5 and 1) and stem length was evaluated (Figure 4). The regenerated shoots were transferred to MS rooting medium containing GA3 (0, 2 and 2.5) and IBA (0, 0.5 and 1) either single or combinations. Next, the plantlets were evaluated for root length and number.

RESULTS AND DISCUSSION

Factorial analysis of variance (ANOVA) test was carried out to detect differences among the factors tested. Cultivars and explants were no significant at 0.005 in all phases except in shoot regeneration which cultivars had significant effect on shooting. Plants growth regulators concentrations had significant effects at 0.005 in all stages (Tables 1, 3 and 5). Callus formation was observed in both explants of hypocotyls and cotyledon tissue segments of the two cultivars of spinach plants, but the percentages of callus formation were not different according to the different explants (Table 1). Our results did not prove Geekiyanage et al. (2006) and Sasaki (1989) results.

In both cultivars, the largest growth of callus occurred on the medium containing 1.5 mgl^{-1} IAA + 2.5 mgl^{-1} GA3. The callus formation in both explants and cultivars was promoted by the presence of GA3 and IAA, addition of GA3 solely to the medium was rather inhibitory to callus formation (Table 2). Our results had some difference with some researchers' results (Al-Khayri et al., 1992; Komai et al., 1996b; Molvig and Rose, 1994; Xiao and Branchard, 1993; Geekiyanage et al., 2006), but confirmed Ishizaki et al. (2000) results. In low IAA and GA3 density, callous formation was dramatically poor, whereas, high density of GA3 and IAA induce callous formation (Figures 1 and 2).

When callus segments of two spinach cultivars were cultured on medium containing various concentrations of GA3 and NAA supplemented, the formation of shoot was obtained by the addition of NAA and GA3. The highest shoot regeneration frequency of 84% was observed from medium containing 0.5 mgl^{-1} NAA + 2 mgl^{-1} GA3 (Table 4). Our result did not prove Kumar et al. (2001) results but, confirm Knoll et al. (1997) results.

The shoot formation was promoted by the presence of both GA3 and NAA and about 68% of the explants formed shoots whereas, approximately 60% callus achieved from Karaj local seedlings had not formed shoots.

On the other hand, the shoot formations were initiated

Table 1. Analysis of variation on the callous growth percentage in all varieties.

S.O.V	DF	SS	MS	F	Probability
Explant	1	0.045	0.045	0.74	$0.3903^{n.s}$
Cultivar	1	0.045	0.045	0.74	$0.3903^{n.s}$
IAA	2	92.480	46.240	764.07	0.0001^{**}
GA3	2	29.161	14.580	240.93	0.0001^{**}
IAA*GA3	4	3.832	0.958	15.83	0.0001^{**}
Error	108	6.536	0.060		

** Significant at 1% probability level, n.s: not significant.

Table 2. Means comparison of callous growth percentage in different hormonal treatments at callous formation stage in all cultivars.

Treatment	Means of callous formation (%)
MS0	0.589 i
2 mgl^{-1} GA3	0.846 h
2.5 mgl^{-1} GA3	1.394 g
1 mgl^{-1} IAA	1.717 f
1 mgl^{-1}IAA +2 mgl^{-1} GA3	2.274 d
1 mgl^{-1}IAA +2.5 mgl^{-1} GA3	2.593 c
1.5 mgl^{-1} IAA	2.019 e
1.5 mgl^{-1}IAA +2 mgl^{-1} GA3	2.974 b
1.5 mgl^{-1}IAA +2.5 mgl^{-1} GA3	3.642 a

Means in each column, followed by at least one letter in common one not significantly different at 5% probability level-using Duncan's multiple range test.

Table 3. Analysis of variation on the stem length in regeneration stage in all varieties.

S.O.V	DF	SS	MS	F	Probability
Explant	1	43.033	43.033	511.75	0.0001^{**}
Cultivar	1	0.019	0.019	0.23	$0.6312^{n.s}$
NAA	2	37.009	18.504	220.05	0.0001^{**}
GA3	2	15.724	7.862	93.50	0.0001^{**}
NAA*GA3	4	3.348	0.837	9.95	0.0001^{**}
Error	108	9.081	0.084		

** Significant at 1% probability level, n.s: not significant.

after about 3 or 4 weeks of incubation. The percentages of bud formation were increased until about 5 weeks after the incubation. As mentioned above in shooting stage cultivars had a significant effect; therefore, callus achieved from Karaj local seedlings had not formed suitable shoots and omitted from investigation (Figure 3).

GA3 is known to promote shoot 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), as shown in our present results. When regenerated shoot segments of spinach plants were cultured on medium containing GA3 and IBA, root formation was observed approximately in all media regardless of the presence of GA3 (Table 6).

Root formation and the taller root in both cultivars occurred on the medium containing 0.5 mgl-1 IBA and application of GA3 inhibited root formation and root elongation (Table 6). Our result did not confirm Geekiyanage and co workers (2006) results.

The inhibition of in vitro root formation by GA3 has been

Table 4. Means comparison of the stem length in different hormonal treatments in regeneration stage in all cultivars.

Treatment	Means of stems length (cm)
MS0	1.153 f
2 mgl^{-1} GA3	1.797 e
2.5 mgl^{-1} GA3	1.319 f
0.5 mgl^{-1} NAA	1.915 d
0.5 mgl^{-1} NAA +2 mgl^{-1} GA3	3.228 a
0.5 mgl^{-1}NAA+2.5 mgl^{-1} GA3	2.708 b
1 mgl^{-1} NAA	1.797 e
1 mgl^{-1} NAA +2 mgl^{-1} GA3	2.392 c
1 mgl^{-1} NAA +2.5 mgl^{-1} GA3	2.131 d

Means in each column, followed by

Table 5. Analysis of variation on root number and length in rooting stage in all varieties.

S.O.V	DF	SS	MF	F	Probability
Root Number					
Cultivar	1	0.0001	0.0001	0.00	$0.9569^{n.s}$
IBA	2	14.592	7.296	143.22	0.0001^{**}
GA3	2	42.663	21.331	418.72	0.0001^{**}
IBA*GA3	4	5.039	1.259	24.73	0.0001^{**}
Error	62	2.859	0.046		
Root Length					
Cultivar	1	0.012	0.0120	0.11	$0.739^{n.s}$
IBA	2	12.082	6.041	56.08	0.0001^{**}
GA3	2	41.204	20.602	191.25	0.0001^{**}
IBA*GA3	4	4.208	1.052	9.77	0.0001^{**}
Error	62	6.678	0.107		

** Significant at 1% probability level, n.s: not significant.

Table 6. Means comparison of the root number in different hormonal treatments in rooting stage in all cultivars.

Treatment	Means of roots number	Means of roots length
MS0	2.592 c	2.550 c
2 mgl^{-1} GA3	1.306 h	1.112 f
2.5 mgl^{-1} GA3	0.483 i	0.619 g
0.5 mgl^{-1} IBA	3.633 a	3.585 a
0.5 mgl^{-1} IBA+2 mgl^{-1} GA3	1.719 f	1.761 d
0.5 mgl^{-1} IBA+2.5 mgl^{-1} GA3	1.306 g	1.235 e
1 mgl^{-1} IBA	2.965 b	2.941 b
1 mgl^{-1} IBA+2 mgl^{-1} GA3	2.282 d	2.343 c
1 mgl^{-1} IBA+2.5 mgl^{-1} GA3	1.991 e	1.828 d

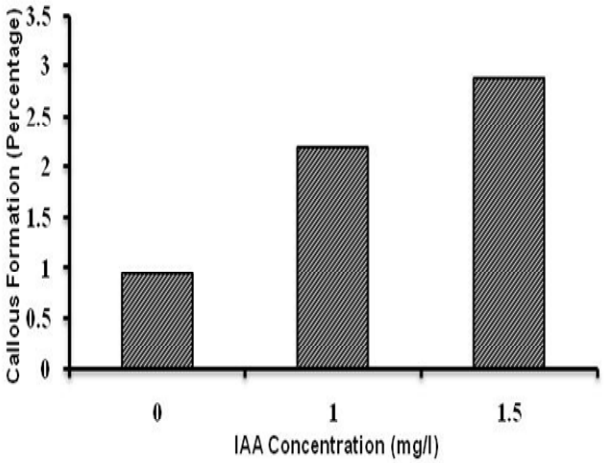

Figure 1. Mean of spinach callous formation in different IAA concentration in all cultivars.

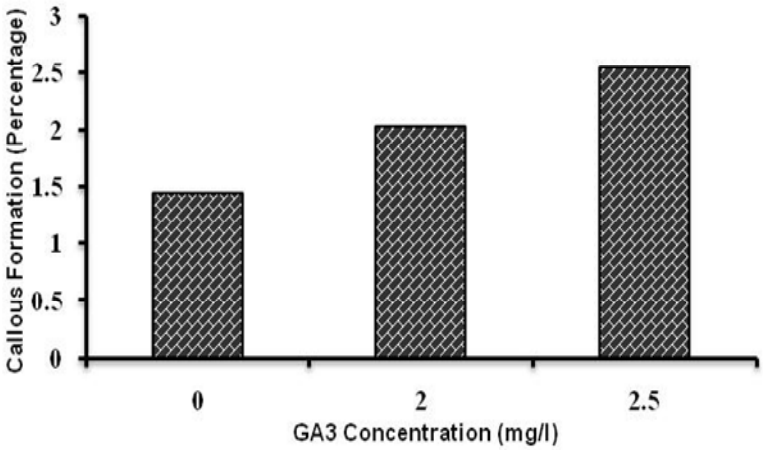

Figure 2. Mean of spinach callous formation in different GA3 concentration in all cultivars.

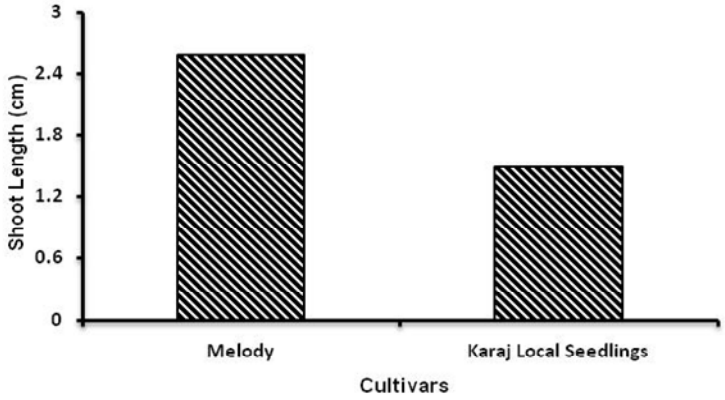

Figure 3. Mean of cultivars shoot length in different hormonal treatments in regeneration stage.

Figure 4. Regenerated plantlet achieved from callous in MS media containing 0.5 mgl^{-1} NAA +2 mgl^{-1} GA3

reported in many plant species (Brian et al., 1955), although, some results were somewhat different depending on the plant materials (Ishizaki et al., 2002). Therefore, the action of exogenous GA3 on root formation of the spinach plant may also be found to differ among the cultivars.

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