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MICROPROPAGATION OF GARLIC CHIVES (*ALLIUM TUBEROSUM* ROTTL. EX SPRANG) USING MESOCOTYL AXIS

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ABSTRACT

Garlic chives - *Allium tuberosum* Rottl. ex Sprang is widely distributed from South East Asia to the Middle East. They are grown for their garlic flavored leaves and are used for culinary purposes. The plants are apomictic, which makes creation of variations and development of new varieties very difficult. The results of previous research show recalcitrant regeneration behaviour in *in vitro* micropropagation cultures. The study was designed to develop efficient regeneration protocols that could help in easy genetic transformation and mutation studies on MS medium containing various concentrations of TDZ-NAA, TDZ-2, 4-D and BAP-IBA using juvenile mesocotyl axis explant obtained from of one week old *in vitro* grown seedlings. The concentrations and combinations of different plant growth regulators in MS medium had significantly different and variable effects on bulblet regeneration. Maximum number of 7.20 shoots per explant was recorded on MS medium containing 1 mg/l BAP-2 mg/l IBA. The shoots regenerated on any regeneration medium were not difficult to root on MS medium containing 0.5 mg/l IBA. These rooted plants were easily acclimatized in the growth chamber. This study clearly shows that mesocotyl axis explants of garlic chives can be successfully cultured on BAP-IBA containing medium to regenerate axillary bulblets. To conclude, this protocol provides a successful and reliable propagation technique to regenerate garlic chive coleoptile axis excised from of one week old *in vitro* regenerated seedlings for the first time. This would help in advanced genetic transformation and mutation studies of the plant in future.

Key words: Garlic chive, mesocotyl axis, *in vitro*, micropropagation, rooting, acclimatization.

INTRODUCTION

The genus *Allium*, belonging to the family Liliaceae, comprises about 700 species, including both economically important vegetables and wild species. *Allium cepa* (onion), *A. sativum* (garlic) and *A. tuberosum* (Chinese chives) are commercially important, other *Allium* species are important locally (Song *et al.* 2007). *A. tuberosum* Rottl. ex Sprang. is widely distributed in South East Asia, South Asia and some countries of the Middle East including Iran that grows at an altitude of 1500-2000 m McGee and Stuckey (2002). Many members of *Allium* are used as food or are important medicinally since thousands of years. They have antimicrobial, antithrombotic, antitumor, hypolipidemic, antiarthritic and hypoglycemic charachteristics related to their high content of organosulfur compounds (Ali *et al.* 2000; Thomson and Ali 2003).

Garlic chives are grown for their leaves, and not for bulbs, which are tough, fibrous elongate and originate from a stout rhizome. Generally its leaves are flat and grass like about 38 cm long 0.8 cm wide and are gray-green in color. The leaves and young inflorescences of garlic chive have garlic like flavor and are used for culinary purposes.

Tissue culture techniques are being increasingly exploited for clonal multiplication and *in vitro* conservation of many *Allium* species including garlic chives (Zee *et al.*1977, Novak et *a1.*, 1986; Shuto, *et al.* 1993, Seabrook 1994, Song and Peffley 1994, Hansen *et al* 1995, Kim and Soh 1996, Matsuda and Adachi 1996, Xue *et al.* 1997, Kim *et al.* 1998, Haque *et al.* 2000, Zhang *et al.* 2004, Mukhopadhyay *et al.* 2005). Breeding plays an important role for *Allium* improvement. Interspecific hybridizations between wild and cultivated species have generated new genotypes possessing biochemical and genetic properties of both parental plants. Some work is also reported on the occurrence of apomictic plants from unpollinated ovule cultures (Kojima *et al.*, 1989), and plantlet regeneration from callus (Zee *et al.*, 1977).

Availability of *in vitro* propagation technique is of particular importance for *A. tuberosum* since the plant is apomictic in nature, which makes creation of variations very difficult. If a suitable regeneration protocol is developed, it will help in permanent transfer of desirable genes in the plants or help in creation of accelerated variations in plants through physical or chemical mutagens that could be employed for improvement of garlic chive against desired traits. The results of previous research show slow developments in *in vitro* micropropagation studies of *Allium* spp in general and

garlic chive in particular. Therefore, there is urgent need to develop efficient regeneration protocols in garlic chive; which will help in transformation, biochemical, molecular and biological studies of *Allium* genes and enzymes easily. In addition, transgenic garlic chives could be used as bioreactors to produce the biochemical compounds that are therapeutically useful (Song *et al.* 2007).

Therefore, the study aimed to find the effects of variants of TDZ-NAA, TDZ-2,4-D and BAP-IBA on bulblet regeneration from juvenile mesocotyl axis tissues excised from of one week old *in vitro* regenerated seedlings of garlic chive, which has not been reported previously.

MATERIALS AND METHODS

The seeds of *A. tuberosum* were collected from Islamic Azad University, Department of Field Crops, Faculty of Agriculture, Miyandoab, Iran. They were surface sterilized in 50% commercial bleach (Ace, Turkey) containing 5-6% NaOCl for 10 min by continuing stirring using a magnetic stirrer. The bleach was then decanted carefully and the seeds were rinsed in double distilled sterilized water. Thereafter, they were cultured on MS medium for germination. Juvenile mesocotyl axis (between the scutellum and the coleoptile) were obtained from one week old germinating seeds and cultured on MS basal medium (Murashige and Skoog 1962) containing 0.75, 1.00 mg/l BAP - 0.25, 0.5, 1 and 2 mg/l IBA or 0.5, 0.75, 1.0 mg/l TDZ-0, 0.01, 0.02, 0.04,0.08 mg/l NAA or . 0.5, 0.75, 1.0 mg/l TDZ - 0, 0.01, 0.02, 0.04,0.08, 0.25, 0.5,1,2,4 mg/l 2,4-D supplemented with 0.65 % agar (Duchefa The Netherlands) and 3.0 % sucrose.

All cultures were incubated in growth chamber at 21±1^{o}C with 16 h light photoperiod. All experimental treatments were performed with six replications.

Regenerated shoots were excised aseptically and rooted on MS medium containing 0.5 mg/l IBA and incubated at 21±1˚C with 16 h light photoperiod. After 4 weeks of culture, agar was carefully removed from the roots and the plants were transferred to pots containing clay, sand and organic matter (1:1:1). Pots were covered with transparent polyethylene bags to maintain the internal humidity and placed in Sanyo versatile growth chamber at 21±1˚C. After one week, transparent polyethylene bags were removed gradually from the pots containing *in vitro* regenerated plants and they were left in the growth chamber with relative humidity of 50%. Thereafter, one week these were transferred to sieved plastic trays containing soil mix (clay, sand and organic matter -1:1:1) and were transferred to cool shady place to bloom and seed.

All treatments of regeneration or rooting experiments had six replicates containing 5 explants each (6 replications x 5 explants = 30 explants). Data for frequency of bulblet regeneration, mean number of bulblets per explant; shoot length and frequency of rooting were recorded and analyzed using one way ANOVA with the help of statistical software SPSS 15.00 for windows. The post hoc tests were performed using Duncan's Multiple Range Test or Turkey's-b test. Data given in percentages were subjected to arcsine transformation (Snedecor and Cochran 1967) before statistical analysis.

RESULTS

Bulblet regeneration from juvenile mesocotyl axis: Juvenile mesocotyl axis tissues showed variable development behavior on each of the MS medium containing various concentrations of TDZ-NAA, TDZ, 2, 4-D and BAP-IBA, which is described as under:

Effects of various concentrations of of TDZ-NAA on bulblet regeneration: Juvenile mesocotyl tissues showed slight swelling before regeneration after 10-11 days of culture. The regeneration medium was not very responsive and the explants showed very recalcitrant regeneration. Reduced mumber of primordia and protuberances of primordia followed by shoot regeneration that would become rhizome shoots were visible on the explants in range of 6.66-76.63% with 0.06-0.86 shoots per explant with mean shoot length of 1-3.46 cm after 8 weeks of culture (Table1).

Effects of various concentrations of of TDZ-2,4-D on shoot regeneration: The explants swelled before regeneration after 9-10 days of culture. Various concentrations of TDZ-2, 4-D affected shoot tegeneration variably. When compared to the previous medium, the regeneration on MS medium containing various concentrations and combinations of TDZ- 2,4-D showed improvement in regeneration. Higher mumber of primordia and primordial protuberances were recorded that followed shoot regeneration that would become rhizome shoots ultimately. No shoot regeneration was recorded on MS medium containing 0.5, 0.75 mg/l BAP or 0.75 mg/l BAP with 2, 4 mg/l 2,4-D. Maximum number of 2.93 shoots were regenerated on MS medium containing 1 mg/l BAP followed closely on MS medium containing 0.5 mg/l TDZ-0.08 mg/l 2,4-D. The results showed maximum shoot length of 5.90 cm on MS medium containing 0.5 mg/l TDZ at the end of 8 weeks of culture (Table2).

Effects of various concentrations of BAP-IBA on bulblet regeneration: Juvenile mesocotyl axis explants elongated and showed slight swelling and development of shoot meristems before regeneration after about 6-7 days of culture; which ended up with miniature shoot like structures after 19-21 days (Fig. 1A). When compared to

previous two mediums containing various concentrations and combinations of TDZ-NAA and TDZ-2,4-D, MS medium containing various concentrations of BAP-IBA. These structures increased in quantity with the passage of time with rhizome like swellings at the base. Maximum mumber of primordia and protuberances of primordia were recorded on the explants that was followed by shoot regeneration. These developed into rhizome shoots on the explants ultimately.

The shoot regeneration frequency on the various concentrations of BAP-IBA was very distinct compared to the plant growth regulator combinations described in Table 1 and 2 in the previous sections. Two concentrations of BAP (0.75 and 1 mg/l) with various combinations of IBA were used in this study. The shoot regeneration frequency on 0.75mg/l BAP- various combinations of IBA and 1mg/l BAP- various combinations of IBA varied significantly such that former (0.75mg/l BAP- various combinations of IBA) was inhibitory in any range of combinations for shoot regeneration percentage, number of shoot per explant and shoot length compared to these charachteristics on the later (1 mg/l BAP- various combinations of IBA).

A general comparison of results on MS medium containing 0.75 mg/l BAP-variants of IBA showed that the shoot regeneration frequency, shoot per explant and their shoot length ranged 13.33-86.66%, 0.13-0.86 and 1-3 cm respectively (Table 3). The maximum shoot regeneration frequency of 86.66%, the maximum number of 0.86 shoots per explant and maximum shoot length of 3 cm was recorded on MS medium containing 0.75 mg/l BAP-0.5 mg/l IBA.

Contrarily, a sharp increase in the shoot regeneration was recorded on MS medium containing 1 mg/l BAP-with various concentrations of IBA. The results showed that the shoot regeneration frequency, shoots per explant and shoot length ranged 93.33-100%, 1.73-7.2 and 4-6 cm respectively. The maximum shoot regeneration frequency of 100% was recorded on 1 mg/l BAP-2-4 mg/l IBA. The shoot regeneration per explant varied inconsistantly on all culture media. The maximum number of 7.20 shoots per explant was recorded on MS medium containing 1 mg/l BAP-2 mg/l IBA (Fig.1B). It was followed by a sharp decrease of 4.40 shoot per explant on MS medium containing 1 mg/l BAP-4 mg/l NAA. Statistically, similar shoot length was recorded on all regeneration media containing 1 mg/l BAP - 0.25 to 2 mg/l IBA, which was followed by a sharp increase in the shoot length on MS medium containing 1 mg/l BAP-4 mg/l NAA.

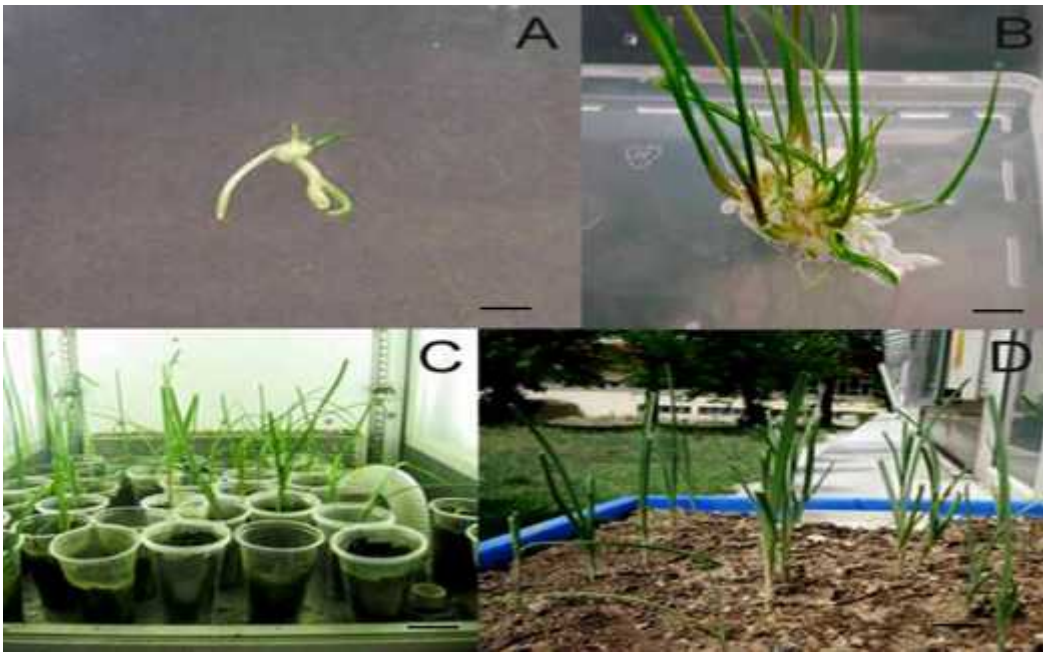

Fig. 1. Shoot regeneration from juvenile mesocotyl axis of garlic chive (A) slight swelling on juvenile mesocotyl axis with regeneration of miniature bulblet like structures after 19-21 days of culture (B) bulblets regeneration on MS medium containing 1 mg/l BAP-2 mg/l IBA (C). *in vitro* regenerated plants acclimatized in the growth chamber at relative humidity of 50 % (D) acclimatized plants transferred to sieved plastic trays containing soil mix transferred to cool shady place to bloom and seed. Bar Fig. 1A=0.3 cm, Fig. 1B=0.5 cm, Fig. 1C=1.2 cm, Fig. 1D=1.5 cm

Rooting and Post acclimatization behavior: The shoots regenerated on any regeneration media were not difficult to root on MS medium containing 0.5 mg/l IBA. These rooted plants were easily acclimatized in the growth

chamber (Fig 1C). When acclimatized plants were transferred to sieved plastic trays containing soil mix (clay, sand and organic matter (1:1:1)) and placed at cool shady place to bloom and seed, they did not show any sign of stress (Fig. 1 D). All *in vitro* regenerated roots decayed in the soil mix (*in vivo*) and developed new profusely branched root apparatus. Survival rate of BAP-IBA regenerated bulblets was 100%. The time required for completing the cycle from the *in vitro* regeneration of bulblets, rooting, acclimatization and transfer to the greenhouse was 16 weeks.

Table 1. Effects of variants of TDZ-NAA on bulblet regeneration from coleoptiles of garlic chive

Treatments		Frequency (%) of bulblet regeneration	Number of bulblets per explant	Shoot length (cm)
TDZ (mg/l)	NAA (mg/l)			
0.5	0.00	43,33 ^{bcd}	0,53 ^{bd}	3,00 ^{ab}
0.5	0.01	16,66 ^{d}	0,26 ^{cd}	3,00 ^{ab}
0.5	0.02	76,63 ^{ab}	0,86 ^{abc}	3,46 ^{a}
0.5	0.04	56,66 ^{b}	0,66 ^{bcd}	3,00 ^{ab}
0.5	0.08	6,66 ^{de}	0,06 ^{d}	1,00 ^{bc}

Values within column followed by different small letters are significantly different at the 0.05 level by Duncans test.

Table 2. Effects of variants of TDZ-2,4-D on bulblet regeneration from coleoptiles of garlic chive

Treatments		Frequency (%) of bulblet regeneration	Number of bulblets per explant	Shoot length (cm)
TDZ (mg/l)	2,4-D (mg/l)			
0.5	0.00	0,00 ^{f}	0,00 ^{g}	0,00 ^{d}
0.5	0.01	76,63 ^{bc}	0,86 ^{def}	3,50 ^{b}
0.5	0.02	76,63 ^{bc}	0,86 ^{def}	5,90 ^{a}
0.5	0.04	56,66 ^{c}	0,66 ^{efg}	3,50 ^{b}
0.5	0.08	96,66 ^{ab}	2,26 ^{ab}	3,00 ^{bc}
0.75	0.00	0,00 ^{f}	0,00 ^{g}	0,00 ^{d}
0.75	0.25	86,63 ^{b}	1,06 ^{de}	1,00 ^{cd}
0.75	0.50	43,33 ^{cde}	0,53 ^{efg}	0,50 ^{c}
0.75	1.00	53,33 ^{cd}	0,60 ^{efg}	0,43 ^{d}
0.75	2.00	0,00 ^{f}	0,00 ^{g}	0,00 ^{d}
0.75	4.00	0,00 ^{f}	0,00 ^{g}	0,00 ^{d}
1.00	0.00	93,63 ^{ab}	1,46 ^{cd}	2,00 ^{bcd}
1.00	0.25	76,63 ^{bc}	0,80 ^{defg}	0,66 ^{d}
1.00	0.50	96,66 ^{ab}	1,80 ^{bc}	1,50 ^{bcd}
1.00	1.00	100,00^{a}	2,93 ^{a}	1,50 ^{bcd}
1.00	2.00	96,66 ^{ab}	1,80 ^{bc}	0,80 ^{d}
1.00	4.00	53,33 ^{cd}	0,60 ^{efg}	1,00 ^{cd}

Values within column followed by different small letters are significantly different at the 0.05 level by Turkey's-b test.

Table 3. Effects of various concentrations of BAP-IBA on bulblet regeneration from coleoptiles of garlic chive

Treatments		Frequency (%) of bulblet regeneration	Number of bulblets per explant	Shoot length (cm)
BAP (mg/l)	IBA (mg/l)			
0.75	0.25	40,00 ^{c}	0,40 ^{f}	2,00 ^{de}
0.75	0.50	86,66 ^{b}	0,86 ^{df}	3,00 ^{cd}
0.75	1.00	13,33 ^{d}	0,13 ^{f}	1,00 ^{ef}
0.75	2.00	20,00 ^{cd}	0,20 ^{f}	1,00 ^{ef}
0.75	4.00	40,00 ^{c}	0,61 ^{f}	1,00 ^{ef}
1.0	0.25	96,63 ^{ab}	1,73 ^{cd}	4,00 ^{bc}
1.0	0.50	93,33 ^{ab}	1,56 ^{de}	4,00 ^{bc}
1.0	1.00	96,66 ^{ab}	2,40 ^{c}	4,33 ^{bc}
1.0	2.00	100,00 ^{a}	7.20 ^{a}	4.33 ^{bc}
1.00	4.00	100.00 ^{a}	4.33 ^{b}	6.00 ^{a}

Values within column followed by different small letters are significantly different at the 0.05 level by Turkey's-b test.

DISCUSSION

Availability of *in vitro* propagation technique is of particular importance for *A. tuberosum* since the plant is apomictic in nature, which makes creation of variations very difficult. If a suitable regeneration protocol is developed, it will help in permanent transfer of desirable genes in the plants or help in creation of accelerated variations in plants through physical or chemical mutagens that could be employed for improvement of garlic chive against desired traits. Several researchers have investigated the proliferation of calli from different explants and plant regeneration from calli with the goal of developing an efficient transformation protocol for onion. Previous results indicate that *Allium* cultures generally grow better in suspension as *Allium* callus grows very slowly on semisolid medium (Zhang *et al.* 2004). Suspension cells that are capable of regeneration have been reported in *A. cepa* (Hansen *et al*. 1995; Mukhopadhyay *et al*. 2005), *A. fistulosum* (Kim and Soh 1996) and an interspecific hybrid (*A. fistulosum* × *A. cepa*; Song and Peffley 1994), however, with very low regeneration capacity. *In vitro* propagation of *A. sativum* has been reported from basal plate or bulb scale explants (Seabrook 1994). Similarly, Haque *et al.* (2000) has reported garlic root tips for micropropagation using variants of BA-NAA.

The concentration of TDZ-NAA, TDZ-2,4-D, BAP-IBA had variable effect on bulblet regeneration. It was observed that various concentrations of TDZ-NAA, TDZ-2, 4-D and also 0.75 mg/l BAP with various concentrations of IBA were inhibitory with much reduced frequency of regeneration and number of bulblets per explant. Matsuda and Adachi (1996) studied the callus formation frequency and plant regeneration capacity of four explant types from four cultivars of Chinese chive. Embryogenic callus was initiated from seedling sections that included the shoot apex part on MS medium supplemented with 5 mg/l 2, 4- 2, 4-D, 3.0% sucrose and 0.8% agar in the light. The best regeneration response (63.8%) was observed on half-strength MS medium supplemented with 1.0 mg/l GA3 and 1.0 mg/l kinetin. Alternatively, plant regeneration from nodular callus was observed in cultures on media with low concentrations of 2, 4-D from all the cultivars. Zhang *et al.* (2002) have also reported efficient plant regeneration via root tip culture on Chinese chive (*Allium tuberosum* Rottle). They showed optimized hormone combination, genotype, seedling age and root induction medium in root tip culture of Chinese chive. Their results showed that the best medium for callus and adventitious shoot differentiation was MS medium supplemented with 1mg/L NAA and 2mg/L BA. They induced shoot induction of 78.7%, 83.7%, 81.9%, 76.7% and 73.3% using cv. Baoding Honggen, Shouguang Malinjiu and Lanzhou Xiaojiu with the average number of 40.1, 46.7, 36.3, 35.4 and 44.5 shoots per explant respectively. When compared with this study the results of Zhang *et al*. (2002) showed a considerable variation. The primary reason to this variation could be difference in the experimental material, explant and the concentrations of plant growth regulators and their combinations. However, the results of this study have edge over the results of Zhang *et al*. (2002); as no callusing was recorded in this study. When the aim is to propagate true to type material, callusing is not desirable, as it leads to somaclonal variations.

Concentration of plant growth regulators in the culture medium had significant influence on adventitious bulblet regeneration. It was noted that BAP-IBA had variable effect on bulblet regeneration such that combination of 0.25-4 mg/l IBA with 0.75 mg/l BAP were extremely inhibitory and combination of same variants of IBA with 1 mg/l BAP were promotory with higher frequency of bulblet regeneration and number of bulblets per explant. This study clearly shows that mesocotyl axis explants of garlic chives can be successfully cultured on BAP-IBA containing medium to regenerate axillary bulblets. Growth regulators dependent differences for plant regeneration have been reported in monocotyledon plants (Luhrs *et al*. 1987, Bregitzer 1992, Hanzel *et al.* 1985, Khawar *et al*. 2005ab, Sevimay *et al*. 2005, Parmaksiz and Khawar 2006, Aasim *et al*. 2008 and Ozel *et al*. 2007, 2008, 2009). The difference in regeneration on different combinations of plant growth regulators are generally attributed to endogenous hormone levels of explants (Morrish, *et al.*1987, Bhaskaran *et al.* 1990) in relation to the competence of mesocotyl axis to regenrate on different concentrations and combinations of TDZ-NAA, TDZ, 2,4-D and BAP-IBA to induce bulblet regeneration.

To conclude, this protocol provides a successful and reliable propagation technique to regenerate garlic chive from juvenile mesocotyl tissues excised from of one week old *in vitro* regenerated seeds for the first time. The study meets objectives and it is postulated that the protocol may be utilized for propagation of other *Allium* species as well.

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