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Adventitious Shoot and Root Regeneration of Wild Strawberry (*F. viridis* Duch.) by Means of Tissue Culture Medium Optimization

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ABSTRACT: The main objective of this study was to clarify the optimum plant growth regulators (PGR) for the organogenesis of wild strawberry. (*Fragaria viridis* Duch.) indigenous to Iran. Nodal segment and stipule were cultured in Murashige and Skoog (MS) containing different concentrations of Cytokinin and Auxin for proliferation and adventitious shoot regeneration respectively. A factorial experiment design in a frame of completely randomize design (CRD) was applied to analyze the data. The MS medium with 8.8 µM N6-benylmino-purine (BA) and 2.5 µM Indole-3- butyric acid (IBA) was the best medium for adventitious shoot regeneration from stipule with producing an average of 6.3 shoot per explants. The highest amount of multiplication was obtained of shoot tip with the average number of 14 regenerated shoots in MS medium containing 2.2 µM Thidiazuron (TDZ) and 0.5 µM IBA. The highest shoot length resulted by the application of a medium containing 2.2 µM BA and 2.5 µM IBA. To accelerate propagation of the adventitious shoot length 0.58 µM Gibberellic acid (GA3) was used. Maximum number of root was derived from 1/2 MS medium contained 1 µM Indole-3- butyric acid. The number of root initiation was decreased with increasing the concentration of Auxin levels in the medium.

Key words: Iranian wild strawberry, organogenesis; micro propagation, TDZ, BA.

INTRODUCTION

Strawberry is one of the popular temperate fruit which always is under cultivation because of its good nutritional properties and flavor. Berry is a valuable fruit for its low calorie, carbohydrate and acceptable fiber contents and is galore in case of antioxidant including phenol, flavonoids, carotenoids, anthocyanin and vitamin C (Larson 1998). All strawberries (*Fragaria* spp.) species have a base haploid count of seven chromosomes (Jijuan *et al.* 2005). *Fragaria viridis* is diploid which have two pairs of chromosomes for a total of 14. This species is distributed at the north area of Iran that produces red and better tasty fruits. However the fruit is smaller than commercial species such as *Fragaria* × *ananassa*. Wild strawberries species are valuable genetic resources in breeding programs for good taste and resistance against biotic and abiotic stresses (Bhatt and Dhar 2000). It propagates naturally by seed and runner that take a long process to fruit and restricted number of seedling respectively. Tissue culture is good substitution for conventionally plant propagation method because of its speed in plant reproduction and introducing new varieties (Taji *et al.* 2002).

Optimization of wild strawberry micro propagation has some benefits and is essential for many purposes such as gene bank, polyploidy, somaclonal variation, protoplast fusion and genetic transformation. Several approaches such as shoot tip culture, adventitious shoot regeneration and somatic embryogenesis are being used for in vitro micro propagation (Passey *et al.* 2003). High efficiency of plant regeneration in tissue culture medium depending on appropriated application of these methods (Cao and Hammerschlag 2000). Many factors such as genotype, culture medium including plant growth regulators (PGR) and their combinations, physical environment and explants effect on regeneration success (Pierik, 1987).

There have been reports for adventitious regeneration from different parts of strawberry, for example from leaves, petioles (Passey *et al.* 2003; Debnath 2005), stem (Graham *et al.* 1995), stipules (Rugini and Orlando 1992), stolons (LIS 1993) and roots (Rugini and Orlando 1992; Passey *et al.* 2003). It is reported that Thidiazuron (TDZ) alone (Debnath 2005) or in combination with 2,4-dichloro-phenoxy-acetic acid (2,4-D) (Passey *et al.* 2003) or Indole-3- butyric acid (IBA) (Yonghua *et al.* 2005) is effective for shoot regeneration in strawberry tissue culture.

Since there are no reports on Iranian wild strawberry propagation, this research was conducted to analysis the effects of explant types and growth regulators on propagation of Iranian wild strawberry (*Fragaria viridis*) in tissue culture medium.

MATERIAL AND METHODS

Just one genotype of *Fragaria viridis* was selected for studied of micro propagation in this paper. This genotype were collected at the north jungle of Iran (Pahneh cola village) with longitude of 53 06, latitude of 36 33 and 327 meters altitude. The shoot tips of runners were separated as explants (size 1 cm) and soak in running tap water for 30 minutes. The explants were transferred to laminar air flow hood and sterilized with ethanol (70%) for 40 seconds, sodium hypochlorite 0.5% for seven minutes and finally in mercuric chloride 0.1% for five minutes. After that the explants were washed with sterile distilled water for three times.

A. Shoot proliferation

Shoot tips (meristematic cells) were prepared and cultured in MS Medium supplemented with B5 vitamins 6-benzylaminopurine (BA 2.2 µM), IBA 0.5 µM and activated charcoal (0.3%) for 2 weeks. The survived explants were sub-cultured in the same medium with different concentration of BA (2.2, 4.4, 8.8, 13.3, 17.7µM), TDZ (0.04, 0.45, 1.1, 2.2, 4.4 µM) and IBA (0.5 and 2.5 µM) for 5 weeks under 3000 lux in 16/8 photoperiod.

B. Adventitious shoot regeneration

Stipules of in vitro plants were utilized for adventitious shoot regeneration with different concentrations of hormones including BA (2.2, 4.4, 8.8, 13.3, 17.7 µM) and IBA (0.5 and 2.5 µM) in MS medium for 4 weeks.

C. Shoot elongation

Micro propagated shoots were cultured in Murashige and Skoog (MS) medium with different concentrations of growth regulators including (MS medium without hormone, 0.13 µM and 0.55 µM Gibberellic acid (GA3) alone and 0.14 µM and 0.58 µM GA3 with 0.88 µM BA) for elongation at 25°C for 4 weeks under 3000 lux in 16/8 photoperiod.

D. Root induction

The obtained shoots were rooted in the ½ MS medium containing different concentration of IBA (0, 1, 2.5, 3.7, 5.7 µM) and 1-Naphthaleneacetic acid (NAA) (0, 1.1, 2.7, 4, 5.4 µM) and maintained in the growth room at 250cwith 16h photoperiod. After 3 weeks rooting percentage, root number and length were counted.

E. Statistical analysis

In shoot proliferation stage all experiments were performed using factorials in a complete randomized design with 6 levels of BA, 4 levels of TDZ in combination with 2 levels of IBA. Of course, factorials experiment in base of complete randomized design (5 levels of BA in combination with 2 levels of IBA four replications per treatment and 4 explants per replication were used in all experiments. Data for each experiment was subjected to analysis of variance (ANOVA) by the General Linear Models procedure using Statistical Analysis Software (SAS) software. Means were compared using the Least Significant Difference (LSD) method, $P \leq 0.05$.

RESULT AND DISCUSSION

Effect of hormones on shoot proliferation: This experiment was conducted to study the effect of Cytokinin (BA, TDZ) and IBA on F. viridis shoot proliferation from shoot tip. All hormonal treatments induced the shoot proliferation in strawberry and significantly differences were observed among treatments in number and length of regenerated shoots. The medium supplemented with 2.2 µM BA and 2.5 µM IBA exhibited after 18 days the latest shoot tip proliferation than other treatments. The MS medium contained 2.2 µM TDZ and 0.5 µM IBA widespread the highest shoot proliferation with 14 shoots from each explants. The proliferated percentage was reduced as TDZ concentration was decreased. Therefore, the MS medium including 0.04 µM TDZ and 0.5 µM IBA had the lowest number of proliferated shoot (Table 1). TDZ exhibited diverse effect on explants in tissue culture medium like shoot proliferation, adventitious shoot regeneration, somatic embryogenesis and callus induction. In some particular woody species in which organogenesis occur hardly, TDZ have improved the in vitro regeneration of these plants like apple (Van Nieuwkerk *et al.* 1986) silver maple (Preece *et al.* 1991), pear (Singha and Bhatia 1988).

Addadi *et al.*, (2010) indicated that TDZ in 1 to 2 mgl^{-1} alone and without combination with BA was the best concentration for proliferation of strawberry cv. Camarosa from nodal segment. Also Debnath (2006) showed that utilization of TDZ in lower concentration of 1 mg^{-1} induced shoot regeneration from sepal. This result approved that the optimum concentration of PGR is so different depending on types of explants and cultivars. In this research TDZ was suitable Cytokinin source for multiplication rather than BA. The number of proliferated shoot was higher in TDZ medium in comparison to the medium with BA. The reason of this result could be the better stability of TDZ rather than all of the Cytokinin hormons (Moke *et al.* 1982). Also Hare *et al.* (1994) reported that the application of TDZ in tissue culture medium increased the internal Cytokinin levels and the reduction of Cytokinin oxidase activation.

Table 1: Effect of Auxin and Cytokinin on shoot proliferation and shoot length of *F. viridis* Data are expressed as means. Means in the same column that are followed by different letters are significantly different (*p*d"0.05) using Duncan's Multiple Range Test.

PGR (µM)		IBA(µM)	Proliferation (%)	Shoot number	Shoot length (cm)
TDZ	0.04	0.5	17	1^{i}	1.5^{dc}
TDZ	0.44	0.5	16	1.3^{hi}	1.4^{dc}
TDZ	1.1	0.5	17	7^{c}	0.5^{g}
TDZ	2.2	0.5	13	14^{a}	0.5^{g}
TDZ	4.4	0.5	8	11^{b}	0.6^{g}
TDZ	0.04	2.5	16	4.3^{d}	1.6^{bc}
TDZ	0.45	2.5	17	6.6^{c}	1.4^{dc}
TDZ	1.1	2.5	14	11.6^{b}	0.7^{ef}
TDZ	2.2	2.5	14	4^{d}	1^{e}
TDZ	4.4	2.5	10	3.6^{d}	0.6^{ef}
BA	2.2	0.5	15	2.3^{fg}	1.6^{bc}
BA	4.4	0.5	15	7.3^{c}	1.4^{bc}
BA	8.8	0.5	16	4^{d}	1.5^{dc}
BA	13.3	0.5	16	3.6^{de}	1.5^{dc}
BA	17.7	0.5	17	3^{ef}	1.4^{d}
BA	2.2	2.5	18	2^{hj}	2^{a}
BA	4.4	2.5	17	3.6^{de}	0.9^{ef}
BA	8.8	2.5	15	4^{d}	0.9^{ef}
BA	13.3	2.5	15	4^{d}	1.7^{b}
BA	17.7	2.5	16	3.8^{d}	1.8^{ab}

Nevertheless, Cytokinin like activity of TDZ, there are some reports on induction of Auxin synthesis and rising of endogenous indole-3-acetic acid (IAA) and tryptophan levels by TDZ application (Murthy *et al.*, 1995). As a consequence, TDZ is a useful candidate for plant organogenesis of many species (Malik and Saxene, 1992). Also the ratio of IBA and TDZ are agents of controlling proliferation efficiency. The highest number of shoots was observed when the TDZ to IBA ratio was 5 fold in tissue culture medium. However, reducing of this ratio caused decline in proliferation. Treatment of 4.4 µM BA along with 0.5 µM IBA was the best treatment among BA containing media (Table 1). Application of lower concentration of BA resulted in a lower shoot number production in culture medium. The best proliferation was observed when ratio of BA to IBA was 10 fold. However, application of other ratio except 10 fold led to less hormone efficiency (Table I). Hormonal balance has a key role in regulation of morphological response from cultured explants. Interaction between Cytokinin and Auxin for plant growth and development is so diverse. This ratio determined the shoot and root formation or efficiency of organogenesis (Landi and Mezzeti 2006).

Data are expressed as means. Means in the same column that are followed by different letters are significantly different ($p \leq 0.05$) using Duncan's Multiple Range Test.

Driven shoot length was different in various culture media in this study. BA in 2.2 µM along with 2.5 µM IBA with 2 cm in shoot length was selected as the best medium and by raising the BA and TDZ concentration, the length of proliferated shoot was decreased gradually (Table I). High concentration of Cytokinin especially TDZ in tissue culture medium had a negative effect in shoot length and regenerated shoot exhibited higher in lower concentration of this PGR. Regenerated shoots from medium containing TDZ in comparison with BA showed different morphological characteristics. TDZ induced compact regeneration and smaller shoots, also leaf blade width was thinner and the color of leaf was lighter in this medium (Fig 1:A-B).

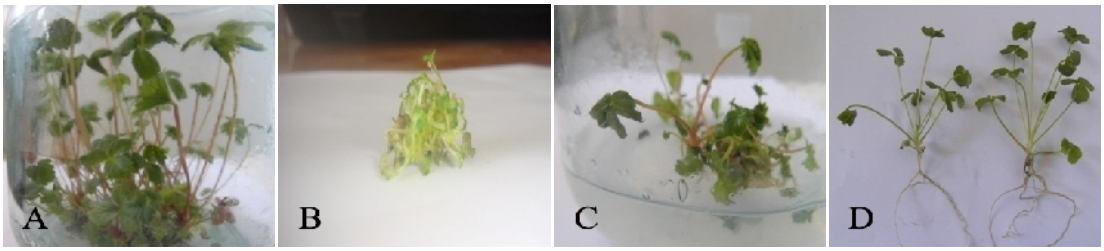

Fig. 1. (A) Shoot tip proliferation from nodal segment of Iranian F. viridis after 5 weeks in MS medium containing 4.4 µM BA and o.44 µM IBA. (B) Shoot tip proliferation from nodal segment of Iranian F. viridis after 5 weeks in MS medium containing 2.2 µM TDZ and 0.5 µM IBA (C) Adventitious shoot regeneration from stipule after 4 weeks of culture in MS medium containing 8.8 µM BA and 0.5 µM IBA. (D) Root induction of proliferated shoot in 1/2 MS medium after 4 weeks in 1 µM IBA.

Also the lowest shoot length (0.5 cm) was observed in MS medium containing 2.2 µM TDZ and 0.5 µM IBA. On the other hand application of Cytokinin alone or without IBA indicated that TDZ had a better potency in shoot proliferation rather than BA. This medium exhibited more shoot proliferation in tissue culture medium from each explants. For shoot elongation, regenerated shoots from the best treatment were transferred to medium containing various hormonal concentrations. Among all of the applied treatments GA in 0.58 µM with 2.5 cm length indicated highest shoots after four weeks. Using BA in combination with GA caused that the length of shoots were smaller than in medium only with GA, but compared with a control the shoots were longer and suitable (Fig. 2). Result showed that the presence of Cytokinin is a preventive factor in shoot elongation medium.

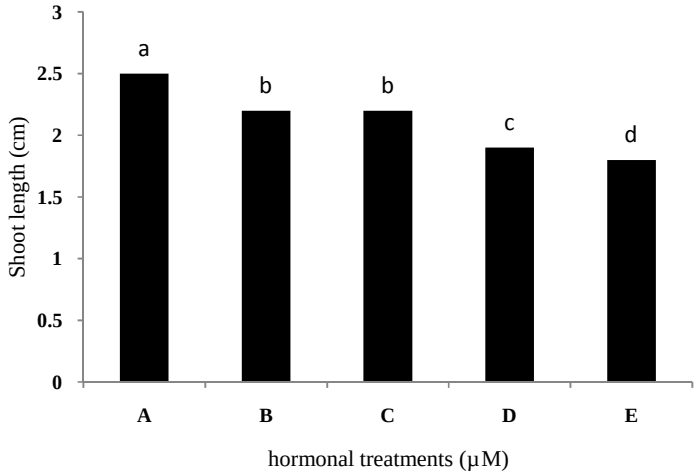

Fig. 2. Effect of different hormonal treatments on shoot length of strawberry in MS medium after 4 weeks.(A= GA3 0.58, B= GA3 0.58 + BA 0.88, C= GA3 0.14 + BA 0.88, D= GA3 0.14, E= Control).

The elongated shoot was cultured in 1/2 MS medium including different concentrations of IBA and NAA for inducing root in regenerated shoot and after 4 weeks. Application of Auxin in moderate concentration improved root number. The efficient medium in aspect of root number production was 1/2 MS medium containing 1 µM IBA. As further concentration of IBA was applied the number of root decreased remarkably (Table 2).

On the other hand, IBA rather than NAA was a better Auxin source for inducing roots. Increasing of NAA up to 5.4 µM caused an inhibition effect concluded root number was significantly reduced (Table 2). However, the best medium for shoot height was control medium. Additionally, by increasing the concentration of NAA and IBA the height of root was decreased. Haddadi et al (2010) reported that the presence of NAA strength the rooting percentage and root number but the medium without any Auxin had the lower number of root. However, the highest root development was observed in the control treatment. Here it was concluded that the root phenotype (number and length) was diverse as influenced by different Auxin treatments.

Table 2: Effect of NAA and IBA in root number and root length of strawberry after 4 weeks in1/2 MS medium.

PGR (µM)	Root number	Root length(cm)
Control	5.3^{de}	7.1^{a}
IBA 1	9.6^{a}	6.7^{a}
IBA 2.5	7^{c}	5^{b}
IBA 3.7	8^{b}	3^{c}
IBA 5	8.6^{b}	3.3^{c}
NAA 1.1	6.3^{cd}	5.3^{b}
NAA 2.7	5.3^{de}	2.8^{c}
NAA 4	4^{f}	2.1^{d}
NAA 5.4	4.3^{ef}	1.1^{e}

It was indicated that a medium containing NAA resulted in thicker roots in contrast to IBA containing medium. Application of NAA in rooting medium induced callus at the end of proliferated shoot and by increasing the NAA quantity the callus production was stimulated remarkably. However, formed root with IBA were thinner and produced more hairy roots in culture. NAA made roots shorter which in turn this condition caused less growth for roots in acclimatization medium.

A. Adventitious shoot regeneration

At the second part of this research stipule explants were cultured in MS medium including different concentrations of BA and IBA for shoot regeneration and after 5 weeks regeneration presence and shoot number were evaluated.

BA as a Cytokinin resource revealed a good potential for inducing direct shoot regeneration in tissue culture medium. The lowest regeneration percentage (10%) was observed in 2.2 µM BA and 2.5 µM IBA but the medium supplemented with 13.3 µM BA and 0.5 µM IBA by 33% indicated the highest regeneration percentage among all media (Fig. 3). For adventitious shoot number, MS medium with 8.8 µM BA and 2.2 µM IBA was selected as a best medium with average of 6.3 adventitious shoot that did not indicated a significantly differences with the medium containing 13.3 µM BA and 0.5 µM IBA with average of 5.3 number (Fig. 3). This part of research is in line with the results of Barcelo *et al* (1998) and SORVARI *et al* (1993) in strawberry.

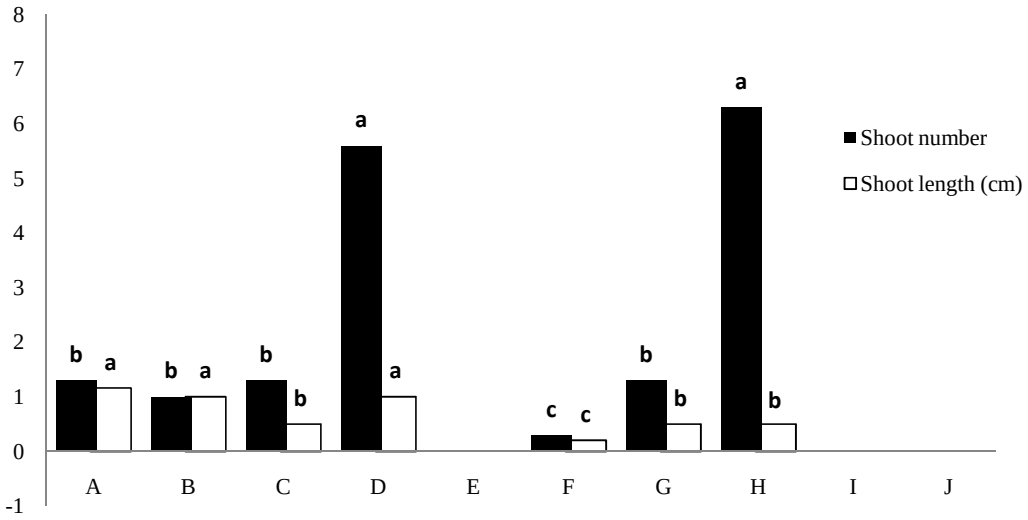

Fig. 3. Effect of hormonal treatments on strawberry adventitious shoots regeneration from stipule after 4 weeks. A: BA2.2+IBA0.5, B: BA4.4+IBA0.5), C: BA8.8+IBA0.5, D: BA13.3+IBA0.5, E: BA17.7+IBA0.5, F: BA2.2+IBA2.5, G: BA4.4+IBA2.5, H: BA8.8+IBA2.5, I: BA13.3+IBA2.5, J: BA17.7+IBA2.5.

Barcelo *et al* (1998) reported that the MS medium containing 8.8 µM BA and 2.5 µM IBA was the best medium for adventitious shoot regeneration from leaf disc of *F.* × *ananassa* cv. `Chandler´. Additionally, the same authors declared that the highest adventitious shoot regeneration in *F.* × *ananassa* cvs. `Hiko´ and `Jonsok´ was obtained in medium was contained with 13.3 µM BA and 0.5 µM IBA. Adventitious shoot regeneration from different parts of strawberry was already reported (PASSEY *et al.* 2003). They announced that among different explants, leaf disc showed the highest regeneration rate. Landy and Mezzeti (2006) studied TDZ and Auxin on shoot regeneration in strawberry leaf and showed that the highest efficiency were obtained from leaf disc by added 1 mgl^{-1} TDZ and 0.5 µM IBA to MS medium rather than other media. On the other hand increased BA concentration reduced the shoot height.

At the lowest concentration of BA and IBA the highest shoot height was observed with 1.16 cm. All treatments showed direct regeneration without callus induction from stipule. Meanwhile some researches explained that callus induction was observed at the margins of leaf explants when the TDZ combined with auxins in comparison with TDZ alone (Landy and Mezzeti 2006). These results confirmed that the genotype is an important factor in explants response and type of organogenesis to plant growth regulators.

CONCLUSION

This research demonstrated that TDZ was an effective Cytokinin among different Cytokinin hormones on shoot regeneration. Also the ratio of TDZ to auxin is another critical issue for high proliferation frequency in short time.

The best shoot multiplication was observed for MS medium containing 4.4 µM TDZ supplemented with 0.5 µM IBA with regenerated 14 shoots in each explants. Raising or decreasing this ratio the number of shoots was reduced visibly. Addition of GA in shoot length medium strength the height of proliferated shoot but existing of BA in this medium had a clear negative effect. In strawberry stipules are suitable explants rather than other tissues for adventitious shoot regeneration. The number and percentage of regenerated shoots is limited in the investigated accession and it seems that the subscription of a special concentration of plant growth regulator is essential for every genotype. Application of 8.8 µM BA plus 2.5 µM IBA give the best adventitious shoot formation from stipule. Rooting of regenerated shoots was observed in all treatments but IBA in 1 µM enhanced the root number and the morphology of roots was more natural and normal type rather than root production in NAA containing medium.

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