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Optimization of *In Vitro* Regeneration System of Newly Developed Strawberry Hybrids

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***Abstract* – *In vitro* techniques are important for clonal multiplication. The clonal propagation of strawberry provides an added advantage for the stable transfer of a desired gene into a commercially important genotype without sexual recombination. The current experiment was conducted to search the most appropriate cytokinine type and the concentrations for *in vitro* propagation of newly strawberry hybrids through cross-breeding and also to examine the effects of auxin and active charcoal (AC) types on rooting stage. Combinations of zeatin and IBA and TDZ and IBA at different concentrations were tested at the micro-propagation stage. For the rooting stage AC, and combination of IBA and AC as well as AC and combination of NAA were tested. Experiment results revealed that the most appropriate concentrations in the propagation stages for each strawberry hybrids were found as follows; 1.0 mg L^{-1} zeatin in combination with 1.0 mg L^{-1} IBA and 1.0 and 1.5 mg L^{-1} TDZ in combination with 1.0 mg L^{-1} IBA. Regarding to rooting stage, AC appeared to be adequate alone instead of using auxin as a supplementary. The highest number of roots and root length per plant were found to be the best only in 3 g L^{-1} AC treatment in the each strawberry hybrids.**

***Keywords* – *In Vitro*, Strawberry, Hybrid, Cytokinin, Auxin, Active Charcoal.**

***Abbreviations Used* – MS: Murashige and Skoog's Medium (1962), Zeatin: 6-[4-hydroxy-3-methylbut-2-enylamino] purine, IBA: Indole-3- butyric acid, TDZ: Thidiazuron; AC: activated charcoal.**

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I. INTRODUCTION

Tissue culture has been extensively used not only in clonal propagation but also in plant breeding studies and also this technique has been applied and found to be suitable nearly for all plants. Meristem culture is regarded as one of the best techniques for micro-propagation of plants that could be easily performed to propagate seedlings properly. This technique is the only way of getting virus-eliminated seedlings [1]. Propagating virus-eliminated seedlings is crucial as it influences both the yield and the quality substantially. Strawberry is propagated vegetatively which, in turn, makes the strawberry plants sensitive to infection, diseases and pests. Therefore, virus-elimination does not appear to be easy. In addition, conventional propagation is highly season-dependent, thereby to get a seedlings from a short-day strawberry, for example in Camarosa cultivar, would be limited under long-day conditions. However, a year-round propagation of strawbery seedlings could be performed by meristem culture and the highest number of seedlings could also be propagated per unit area. Another advantage of meristem culture is that this technique enables one to maintain the gene-resources and to facilitate propagation at short intervals of time [2, 3, 4, 5]. *In vitro* techniques are important tools for modern plant improvement programs introducing new traits into selected plants, to multiply elite selections and to develop suitable cultivars in the minimum time. Used in conjunction with classical breeding methods, an efficient *in vitro* shoot proliferation and regeneration system could accelerate cultivar development programs [6].

A current experiment showed that the plants propagated in meristem culture could be transferred to the soil in 12 weeks after meristem growth and development stages and would be ready for field conditions in 2 months after transferring to soil [7]. Several studies have attested that the tissue cultured plants have an advantage over those obtained by conventional propagation in terms of fruit yield [8, 9, 10, 11, 12], pest resistance, vigor, yield per plant, the number of runners and leaves per plant [13, 14, 15]. Micropropagated strawberry 'Gorella' showed higher resistance to frost damage than did standard runner plants, when injury was evaluated in the field in spring [16]. Hormone type, its concentration in propagation and root induction and so also the cultivar are important factors influencing the success of *in vitro* culture [17, 18]. Considering the cultivars, the success of meristem culture in Seascape cultivar was 10%, whereas it was nearly 90% in Camarosa and Albion cultivars. [19]. Cytokinins play an important role in enhancing the propagation and also the shoot quality. Further, auxins too are as influential as the cytokinins, on propagation, to some extent [20]. Hence, the most important aspect is to determine the appropriate hormone type and to use the same in optimum concentrations. The hormones, BAP, IAA, IBA and TDZ have been extensively used is strawberries [21, 22]. One disadvantage of TDZ is its often inhibitory effects on shoot elongation as demonstrated for several woody species [23].

Auxins and cytokinins used in nutrient solution has simulative effects on the tissue development. TDZ is responsible for biosynthesis of cytokinin and preserves endogenous hormones in plant tissues [24]. At the same time, TDZ reduces ethylene production and promotes cell division in *in vitro* culture [25]. Many scientist have reported the use of TDZ for shoot regeneration from leaf discs in strawberry [26, 27, 28]. Cytokinins such as 6-[4-hydroxy-3-methylbut-2-enylamino] purine (zeatin),

thidiazuron (TDZ) and auxins indole-3-butyric acid (IBA) and indole-3-acetic acid (IAA) have also been reported for shoot proliferation [29]. Zeatin was found to be effective for shoot initiation in Vaccinum species [30], and shoot proliferation of lingonberry [31].

In tissue culture, auxins are used widely at the rooting stage and the type and concentration of auxins plays an important role in successful rooting. Some auxin types influence callus formation or rooting depending on the plant type and species. Some studies have shown that NAA enhances callus formation rather than rooting as compared to IBA [32, 7]. Further, higher concentrations of auxins promotes callus formation, thus leading to less root formation [33]. In addition to auxins, supplemental active charcoal (AC) enhances root formation in some species and also preserves some taxa from phenolic compounds [34, 35]. Shaakeel ve Iqtiar [36] studied Chandler cultivar in meristem culture and found that there is no need to use of hormone or use IBA as low as (0.1 mg L^{-1}) to induce rooting.

In this experiment aims to determine the best hormone type, and optimum concentrations and combinations on 'TP14-22-20', 'TP14-2-84' and 'TP14-14-6' strawberry hybrids.

II. Materials and Methods

A. *Plant Material and Experimental Conditions*

In this experiments, three hybrids of strawberry obtained through traditional breeding method by Teydep-Turkey Project No. 711143 ('TP14-22-20', 'TP14-2-84', 'TP14-14-6') were used as test plants and those shoot tip from primer runners collected in May were used as explants. The shoot tips were surface sterilized by keeping the min 96% alcohol for 5-10 seconds under sterile conditions followed by two-step sterilization processes were carried out. The explants were exposed to %15 sodium hypochlorite for 15 minutes, the outer leaves were removed and the shoot apices were treated with 5% sodium hypochlorite for 5 minutes to ensure complete surface sterilization. Following sterilization processes, all the explants were inoculated on media [7]. Murashige and Skoog [37] (MS) medium with 3% sucrose and 0.2% Phytagel were used. Meristems were isolated under binocular microscope to possess one or two leaf primordia and were cultured on MS medium for 4 weeks [38]. 1 mg L^{-1} BAP and 1 mg L^{-1} IAA combinations were tested for meristem growth and development stage. The following treatments were used for propagation;

a) 0, 0.5, 1.0, 1.5 and 2.0 mg L^{-1} Zeatin + 1 mg L^{-1} IBA.
b) 0, 0.5, 1.0, 1.5 and 2.0 mg L^{-1} TDZ + 1 mg L^{-1} IBA.

Observations were recorded for number of shoots produced per explants and the quality of the shoots produced following treatment after three sub-cultures.

The treatments given in the rooting stage were:

a) AC (3 g L^{-1}), 0.1, 0.4, 0.8 and 1.0 mg L^{-1} IBA + AC (3 g L^{-1}).
b) AC (3 g L^{-1}), 0.1, 0.4, 0.8 and 1.0 mg L^{-1} NAA + AC (3 g L^{-1}).

These treatments were evaluated for number of roots, root length, leaf number and stem diameter before transferring the plants to field.

In the culture room, temperature was maintained at 25°C±1ºC, light intensity was 3000 µmol $s^{-1}m^{-2}$. All treatments were conducted a 3 replicates, 10 jar in the propagation stages and 2 explants in each. In the rooting stage 15 jars and 1 explant for each were used.

B. *Statistical Methods*

The study was carried out in randomized parcels design is statistically and LSD test was used for the comparisons.

III. Results and Discussions

A. *Evaluation of Propagation Stages*

Zeatin + IBA Concentrations

Table 1 gives the results of different zeatin concentrations in combination with 1 mg L^{-1} IBA on the shoot number per explants in 'TP14-22-20'. Clearly, 1 mg L^{-1} zeatin with 1 mg L^{-1} IBA gave the highest (21.94 shoots/explant) and control brought about the lowest shoot number (4.22 shoots/explant). In addition, shoot quality and number showed a decrease in more or less than 1 mg L^{-1} Zeatin concentrations.

In 'TP14-2-84', different Zeatin concentrations in combination with 1 mg L^{-1} IBA were found to be statistically significant on the shoot number per explants as for 'TP14-22-20'. Zeatin concentrations more than 1 mg L^{-1} caused shoot number to decrease and to get thinner and to decline in shoots (Table 1). To 'TP14-4-6', the same results were obtained with the others, different Zeatin concentrations in combination with 1 mg L^{-1} IBA and the highest shoot number was taken from 0.5 and 1.0 mg L^{-1} Zeatin combinations, while the lowest from control.

In conclusion, the highest shoot number in each hybrid was obtained in 1 mg L^{-1} Zeatin and in combination with 1 mg L^{-1} IBA. Propagation capacity in these concentrations was found to be greater and ranged from 21.94 and 23.19 shoots per explants. Zeatin concentrations higher or lower than 1 mg L^{-1} brought about a decline in shoot number and quality. Marcotrigiano et al. [39] stated optimum BAP concentrations to be in the range of 1-3 mg L^{-1} and 0.3 mg L^{-1} in propagation stage; however, Margara [40] pointed out the most suitable BAP concentrations to be 1 mg L^{-1}. Further, Merkle [22] cited that 1.0 mg L^{-1} BAP and 0.5 mg L^{-1} IBA or 2.0 mg L^{-1} BAP and 2.0 mg L^{-1} IBA combinations would be the best option for shoot propagation. Ko *et al.* [41] have observed that MS medium supplemented with 1.0 mg L^{-1} BA and 0.1 mg L^{-1} NAA gave better results as compared to 5 mg L^{-1} BA and 0.02 mg L^{-1} NAA with respect to shoot regeneration in strawberry. These differences were most probably because of different genotypes. But there haven't found studies related to zeatin in propagation stages on strawberry. Zeatin was found to be effective for shoot initiation in Vaccinum species [30]. All studies clearly reveal that cytokinins should be used in combination with auxins. In addition, cytokinins and auxins ratio should be 1 or more in order to make propagation capacity higher.

TDZ + IBA Concentrations

The results of different TDZ concentrations in combination with 1 mg L^{-1} IBA are given in Table 2. TDZ concentrations had a significant effect on shoot number per explants and the highest number was obtained in 1 and 1.5 mg L^{-1} TDZ in

Table 1. Shoot numbers in 'TP14-22-20', 'TP14-2-84' and 'TP14-14-6' with regard to different Zeatin concentrations in combination with 1 mg L^{-1} IBA

Hybrids	Treatments (mg L^{-1})	No. of shoots (shoots/explant)
TP14-22-20		
	Control (MS)	4.22 d *
	0.5	17.10 b
	1.0	21.94 a
	1.5	15.20 b
	2.0	10.35 c
	LSD$_{\%5}$	***2.365***
TP14-2-84		
	Control (MS)	4.69 d
	0.5	17.55 ab
	1.0	23.02 a
	1.5	18.19 b
	2.0	10.06 c
	LSD$_{\%5}$	***4.583***
TP14-14-6		
	Control (MS)	4.89 d
	0.5	22.21 ab
	1.0	23.19 a
	1.5	19.16 b
	2.0	10.17 c
	LSD$_{\%5}$	***2.045***

*Means followed by the same letters within columns are not significantly different at $LSD_{\%5}$

combination with 1 mg L^{-1} IBA, while the lowest was in control. In 'TP14-2-84', 1.5 mg L^{-1} TDZ and 1 mg L^{-1} IBA combinations gave the maximum shoot number with 28.38 shoots per explants, followed by 1 mg L^{-1} TDZ and 1 mg L^{-1} IBA combinations with 26.52 shoots per explants. 'TP14-14-6' nearly gave the same results like the others, but at TDZ concentrations greater than 1.5 mg L^{-1} and less than 1 mg L^{-1} gave rise a decline in shoot number and quality and control gave also the less shoot number (Table 2).

This work proved that TDZ addition had a positive effect on shoot propagation capacity and quality. The most suitable concentrations were determined to be 1 and 1.5 mg L^{-1} TDZ in combination with 1 mg L^{-1} IBA. These concentration produced shoot number in higher quantity, ranging from 25.23 and 28.33 shoot per explants. Therefore, the superiority of TDZ to BAP was clear in terms of shoot regeneration. Nonetheless, Wickremasinghe and Fernando [21] were found BAP to be superior to TDZ in propagation stages in enhancing propagation capacity and shoot quality. Adak *et al.* [42] reported; however, that TDZ usage in combination with IAA was beneficial for propagation in strawberries. Some researchers found that the combination of TDZ and IBA increased the proliferation of strawberry. In those study, keeping TDZ at 1.5 mg L^{-1}, an increase of IBA concentration from 0.2 to 0.4 mg L-1 significantly increased regeneration efficiency in terms of both percent of shoot regeneration and number of shoots per explants [27]. Qin *et al.* [43] studied regeneration by strawberry leaf disc in MS medium and found that 1.5 mg L^{-1} TDZ and 0.4 mg L^{-1} IBA gave the better regeneration. However, differences among the concentrations might possibly be related to the explants type and also different genotype, but 1.0 and 1.5 mg L^{-1} TDZ were determined to be the sufficiency levels.

Table 2. Shoot numbers in 'TP14-22-20', 'TP14-2-84' and 'TP14-14-6' with regard to different TDZ concentrations in combination with 1 mg L^{-1} IBA

Hybrids	Treatments (mg L^{-1})	No. of shoots (shoots/explants)
TP14-22-20		
	Control (MS)	4.22 c*
	0.5	17.45 b
	1.0	26.32 a
	1.5	27.95 a
	2.0	19.10 b
	LSD%5	3.353
TP14-2-84		
	Control (MS)	4.69 c
	0.5	17.35 b
	1.0	25.29 a
	1.5	29.39 a
	2.0	19.76 b
	LSD%5	2.821
TP14-14-6		
	Control (MS)	4.89 c
	0.5	19.15 b
	1.0	25.25 a
	1.5	27.32 a
	2.0	19.31 b
	LSD%5	2.375

*Means followed by the same letters within columns are not significantly different at $LSD_{\%5}$

B. Evaluation of Rooting Stages

IBA +AC Concentrations

Different concentrations of IBA in combination with AC showed a significant effect on root number in different strawberry hybrids (Table 3). In 'TP14-22-20', the highest root number was observed in 3 g L^{-1} AC, having 9.57 roots per plant. The higher the IBA concentration applied, the less was the root number produced. The longest root length was also taken in 3 mg L^{-1} AC with 6.65 cm and followed by 0.1 mg L^{-1} IBA + AC (5 g L^{-1}) with 6.43 cm. The shorter root length was observed in the plants treated with 1.0 mg L^{-1} IBA + AC (3 g L^{-1}). In addition, the highest leaf number per plant was achieved only in AC; while elevated IBA gave rise to a decline in leaf number. Regarding to stem diameter, an insignificant response was fixed among the treatments.

In 'TP14-2-84', a significant relation was recorded with regard to treatments, and AC gave the better results as determined in 'TP14-22-20'. The highest root number was similarly found in AC and elevated concentrations of IBA caused a decrease on those as the same as 'TP14-22-20'. The higher AC and the lowest IBA resulted in the longest length. The highest leaf number was observed only in AC, whereas stem diameter showed statistical significance among the treatments. Similar results were recorded for 'TP14-14-6'; however, the highest number of root, root length and leaf number were found only in AC. Regarding to IBA + AC, elevated IBA concentration had led to a decrease on those parameters.

In summary, the use of AC in rooting stage was beneficial for rooting, plant growth and development; however, elevated concentrations of IBA in AC medium caused rooting capacity to decrease. Thus, if AC and IBA are to be used, 0.1 mg L^{-1} IBA in AC medium was found to be most suitable dose. The higher the IBA concentration used, the more was the callus formed which it's not desired in meristem culture. These findings are in agreement with those of Çömlekçioğlu and Kaşka [44] addressing the beneficial effects of AC usage in rooting stages. Moreover, Lopez *et al.* [11] stated that a supplemental AC addition, 0.5 g L^{-1}, was sufficient for proper rooting.

NAA + AC Concentrations

In 'TP14-22-20', a statistical significance was recorded in NAA treatment and its combination with 3 g L^{-1} AC in terms of root number per plant (Table 4). The highest root number was 8.47 per plant in just 3 g L^{-1} AC treatment followed by 0.1 mg L^{-1} NAA+AC (3 g L^{-1}) and 0.4 mg L^{-1} NAA+AC (3 g L^{-1}), resulting in 5.33 and 4.53 roots per plant. The longest root and the highest leaf number were detected in only AC treatment, while stem diameter response was insignificant to the treatments.

Regarding 'TP14-2-84', AC gave the best results in root length, root number and leaf number, but stem diameter was not influenced by the treatments statistically. The higher the NAA concentration, the lower were the measured parameters observed (Table 4). Moreover, callus formation was noticed in elevated NAA treatments.

In 'TP14-14-6', different NAA and AC combination were found to be statistically significant on root number, root length and leaf number, while stem diameter was not found to be statistically significant (Table 4). The highest root number was 8.30 roots per plant in AC (3 g L^{-1}), and the lowest was from 1.0 mg L^{-1} NAA+AC (5 g L^{-1}) with 3.40 roots per plant. In addition, AC (3 g L^{-1}) also gave the highest root length with 6.60 cm, whereas 1.0 mg L^{-1} NAA+AC (3 g L^{-1}) resulted in the lowest number of roots with 3.13 cm.

Finally, NAA + AC treatments showed similar response as received from IBA + AC and an elevation in NAA concentration increased the callus formation and decreased the rooting capacity as well. Therefore, only AC was found to be effective in terms of rooting, plant growth and development. These findings are in agreement with Waithaka *et al.* [32], indicating callus formation enhancement in the supplemental addition of 1 mg/l NAA to the medium.

IV. Conclusions

In conclusion, for shoot propagation, the most suitable Zeatin concentrations was 1 mg L^{-1}, and TDZ was 1mg L^{-1} and 1.5 mg L^{-1} in combination with 1 mg L^{-1} IBA. Further, concentrations more than 1 mg L^{-1} Zeatin and than1.5 mg L^{-1} TDZ caused negative affect on shoot propagation and shoot quality. In rooting stage, AC utilization rather than auxins had a positive influence on rooting, plant growth and development. NAA enhanced callus formation and depressed the rooting capacity; thus, elevated concentration of NAA could be used in breeding studies for variation.

In clonal propagation, 1 mg L^{-1} Zeatin + 1 mg L^{-1} IBA and 1 mg L^{-1} TDZ + 1 mg L^{-1} IBA in propagation stages and 3 g L^{-1} activated charcoal in the rooting stages were found to be the best concentrations. The results of the current experiment would be helpful for breeding studies by shortening the breeding time; thus, new varieties could be achieved in short intervals.

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Table 3. Number of roots, root length, number of leaves and stem diameter in 'TP14-22-20', 'TP14-2-84' and 'TP14-14-6' with regard to different IBA concentrations in combination with 3 mg L^{-1} AC

Hybrid/Treatments	No. of roots (root/plant)	Root length (cm)	No.of leaves (leaf/plant)	Stem diameter (mm)
TP14-22-20				
AC (3 g L^{-1})	9.57 a*	6.65 a	10.17 a	4.12
0.1 mg L^{-1} IBA+AC (3 g L^{-1})	7.65 b	6.45 a	9.62 a	4.15
0.4 mg L^{-1} IBA+AC (3 g L^{-1})	7.25 b	6.21 ab	8.25 b	4.01
0.8 mg L^{-1} IBA+AC (3 g L^{-1})	5.85 c	5.55 b	7.75 b	4.00
1.0 mg L^{-1} IBA+AC (3 g L^{-1})	5.55 c	4.61 c	7.67 b	3.95
***LSD*$_{\%5}$**	***0.572***	***0.799***	***1.054***	***N.S.*****
TP14-2-84				
AC (3 g L^{-1})	9.23 a	6.53 a	10.11 a	4.12
0.1 mg L^{-1} IBA+AC (3 g L^{-1})	7.74 b	6.49 a	9.42 b	4.11
0.4 mg L^{-1} IBA+AC (3 g L^{-1})	7.42 b	6.25 a	8.62 c	3.89
0.8 mg L^{-1} IBA+AC (3 g L^{-1})	6.21 c	5.36 b	8.21 c	3.82
1.0 mg L^{-1} IBA+AC (3 g L^{-1})	5.85 c	4.33 c	7.92 c	3.81
***LSD*$_{\%5}$**	***0.690***	***0.499***	***0.751***	***N.S.***
TP14-14-6				

AC (3 g L^{-1})	8.65 a	6.55 a	9.75 a	4.09
0.1 mg L^{-1} IBA+AC (3 g L^{-1})	7.46 b	6.33 a	9.73 a	4.10
0.4 mg L^{-1} IBA+AC (3 g L^{-1})	7.33 b	6.17 a	8.77 b	3.99
0.8 mg L^{-1} IBA+AC (3 g L^{-1})	5.95 c	5.25 b	7.01 c	3.89
1.0 mg L^{-1} IBA+AC (3 g L^{-1})	5.55 c	4.65 b	7.62 bc	3.71
$LSD_{\%5}$	***0.695***	***0.636***	***1.023***	***N.S.***

*Means followed by the same letters within columns are not significantly different at $LSD_{\%5}$

Table 4. Number of roots, root length, number of leaves and stem diameter in 'TP14-22-20', 'TP14-2-84' and 'TP14-14-6' with regard to different NAA concentrations in combination with 3 mg L^{-1} AC

Hybrid/Treatments	No. of roots (root/plant)	Root length (cm)	No. of leaves (leaf/plant)	Stem diameter (mm)
TP14-22-20				
AC (3 g L^{-1})	9.57a*	6.65 a	10.17 a	4.12
0.1 mg L^{-1} NAA+AC (3 g L^{-1})	5.31 b	5.10 b	5.12 b	4.10
0.4 mg L^{-1} NAA+AC (3 g L^{-1})	4.55 c	5.10 b	5.02 b	4.00
0.8 mg L^{-1} NAA+AC (3 g L^{-1})	4.15 c	3.71 c	5.00 b	4.15
1.0 mg L^{-1} NAA+AC (3 g L^{-1})	3.34 d	3.23 c	4.00 c	3.71
$LSD_{\%5}$	***0.485***	***0.625***	***1.426***	***N.S.****
TP14-2-84				
AC (3 g L^{-1})	9.23 a	6.53 a	10.11 a	4.12
0.1 mg L^{-1} NAA+AC (3 g L^{-1})	6.32 b	4.95 b	5.06 b	4.11
0.4 mg L^{-1} NAA+AC (3 g L^{-1})	6.31 b	4.32 c	5.03 b	4.02
0.8 mg L^{-1} NAA+AC (3 g L^{-1})	5.55 c	3.12 d	5.07 b	4.01
1.0 mg L^{-1} NAA+AC (3 g L^{-1})	4.32 d	3.03 d	4.31 c	4.02
$LSD_{\%5}$	***0.392***	***0.368***	***0.518***	***N.S.***
TP14-14-6				
AC (3 g L^{-1})	8.65 a	6.55 a	9.75 a	4.09
0.1 mg L^{-1} NAA+AC (3 g L^{-1})	4.21 b	5.25 b	5.37 b	4.03
0.4 mg L^{-1} NAA+AC (3 g L^{-1})	4.11 bc	5.11 b	5.12 b	4.01
0.8 mg L^{-1} NAA+AC (3 g L^{-1})	3.66 bc	3.32 c	4.82 b	4.01
1.0 mg L^{-1} NAA+AC (3 g L^{-1})	3.45 c	3.21 c	4.72 b	4.00
$LSD_{\%5}$	***0.758***	***0.456***	***0.632***	***N.S.***

*Means followed by the same letters within columns are not significantly different at $LSD_{\%5}$

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