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# Chitosan and thidiazuron improve regeneration efficiency of strawberry (*Fragaria* x *ananassa* Duch.) cv. Festival from different explant types

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## ABSTRACT

Establishment of an efficient regeneration system is considered as a prerequisite step for genetic transformation and somaclonal variation which have provided alternative and efficient strategies to the breeders for obtaining genetic variability and improving new genotypes. To optimize the regeneration protocol for strawberry cv. Festival, different explant types, i.e. leaf discs, petioles, stipules, and roots were cultured on $N_{30}K$ mineral formulation with MS microelements medium containing different concentrations of indole-3-butyric acid (IBA) (0.2 or 0.5 mg $l^{-1}$) with thidiazuron (TDZ) (1.0, 1.5 or 2.0 mg $l^{-1}$) or 6-benzylaminopurine (BA) (1.0, 2.0 or 3.0 mg $l^{-1}$) and supplemented with different concentrations of chitosan (0, 25 or 50 mg $l^{-1}$). In addition, the effect of chitosan on shoot multiplication was tested. Results clearly revealed that leaf discs proved to be a good source of explant material for regeneration of Festival cultivar, since the regeneration efficiencies varied from 58 to 100%, from 0 to 40%, from 8.33 to 47.66%, and from 11 to 57.66%, for leaf discs, petioles, stipules, and roots, respectively. In addition, TDZ was more effective than BA and chitosan application acted as a growth stimulator for shoot regeneration from all tested explants. A medium containing 0.5 mg $l^{-1}$ IBA and 1.5 mg $l^{-1}$ TDZ supplemented with 25 mg $l^{-1}$ chitosan was found to be the most efficient combination for the regeneration from leaf discs, petioles and stipules, and produced the highest number of shoots/leaf disc. The medium containing 0.5 mg $l^{-1}$ IBA and 2.0 mg $l^{-1}$ TDZ supplemented with 50 mg $l^{-1}$ chitosan produced the highest number of shoots regenerated from petioles and stipules, while 0.5 mg $l^{-1}$ IBA and 2.0 mg $l^{-1}$ TDZ combination with 25 mg $l^{-1}$ chitosan gave the highest regeneration frequency and number of shoots for roots. Moreover, chitosan application at 25 or 50 mg $l^{-1}$ had positive effect on shoot multiplication. Both concentrations accelerated the appearance of the proliferation rate by 5-7 days and increased number of plantlets/explant without significant differences. In conclusion, an efficient regeneration protocol was optimized for strawberry cv. Festival from different explant types.

***Key words:*** *In vitro*, Organogenesis, Adventitious shoot regeneration, Thidiazuron

## Introduction

Strawberry, *Fragaria* x *ananassa* Duch., is an important berry fruit cultivated in the temperate and sub-tropical climates including Egypt. Production of new cultivars is a continuous process to meet the demands for improved yields and quality traits. However, the narrow genetic base of the cultivated strawberry associated with high heterozygosity and polyploidy hamper traditional breeding methods. Biotechnological approaches, especially genetic transformation and somaclonal variation, have provided alternative and efficient strategies to the breeders for obtaining genetic variability. In this connection, establishment of an efficient regeneration system in strawberry, especially for new cultivars introduced to Egypt such as Festival cultivar, is considered as a first and a prerequisite step for both approaches.

Regeneration *via* adventitious organogenesis has been described in many strawberry cultivars and the previous studies have clearly demonstrated the importance of several factors such as genotype (Passey *et al.,* 2003), explant type (Nehra *et al.*, 1989), hormonal balance (Barceló *et al.*, 1998), previous cultural conditions of the donor plants (Monticelli *et al.*, 2002), incubation conditions (Nehra *et al.*, 1989), and some other growth stimulators like casein hydrolysate (Liu and Sanford, 1988) on the success of the regeneration. These diverse factors affecting genotype-specific regeneration make standardization of an efficient regeneration system for each strawberry genotype an indispensable

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prerequisite. In this study, type of explant, auxin and cytokinin balance, and chitosan (as a growth stimulator) were studied to optimize the regeneration protocol for Festival cultivar.

Explant types have been aroused an extensive interest as one of the key factors that affects the regeneration efficiency. In this, respect, adventitious shoot regeneration of strawberry has been accomplished from a range of *in vitro* cultured explants such as leaf discs (Passey *et al.*, 2003; Husaini and Srivastava, 2006; Husaini and Abdin, 2007; Ara *et al.*, 2012; Mahmoud and Kosar, 2013; Nasri and Bahramnejad, 2013; Cappelletti *et al.*, 2016), petioles (Rugini and Orlando, 1992; Damiano *et al.*, 1997; Passey *et al.*, 2003; Mezzetti *et al.*, 2004; Folta *et al.*, 2006), stipules (Rugini and Orlando, 1992; Damiano *et al.*, 1997; Monticelli *et al.*, 2002; Passey *et al.*, 2003; Sönmez and Kafkas, 2012), roots (Rugini and Orlando, 1992; Passey *et al.*, 2003), and others. However, previous studied stated that the highest regeneration efficiency was achieved using leaf discs and petioles as explants (Liu and Sanford, 1988; Nehra *et al.*, 1989; Jelenkovic *et al.*, 1990; Popescu *et al.*, 1997; Passey *et al.*, 2003). Nevertheless, strawberry cultivars differently responded to the range of regeneration protocols due to their diverse genetic backgrounds (Passey *et al.*, 2003).

Efficient organogenesis also depends on the concentrations of plant growth regulators in the culture medium, and the response varies according to the explant type and cultivar (Zakaria *et al.*, 2014). A combination of types and levels of auxin and cytokinin generally is necessary for successful regeneration (Motte *et al.*, 2014). Indole-3-acetic acid (IAA) and 6-benzylaminopurine (BA) combination gave the highest regeneration capacity in Redcoat (Nehra *et al.*, 1989), and Sweet Charli and Pajaro cultivars (Singh and Pandey, 2004), while indole-3-butyric acid (IBA) and BA gave the best results in the regeneration of Chandler (Barceló *et al.*, 1998; Husaini and Srivastava, 2006), and 2,4-dichlorophenoxyacetic acid (2,4-D) and BA was the best combination for Totem and Hood regeneration (Finstad and Martin, 1995). Recently, there has been an increased interest in the use of thidiazuron (TDZ), 1-Phenyl-3-(1,2,3-thiadiazol-5-yl) urea, in strawberry regeneration. Many studies have revealed that TDZ was very effective for promoting shoot regeneration of strawberry from leaf disks (Schaart *et al.*, 2002; Passey *et al.*, 2003; Zhao *et al.*, 2004; Qin *et al.*, 2005; Landi and Mezzetti, 2006; Husaini and Abdin, 2007), stipules (Sönmez and Kafkas, 2012), and sepals and petioles (Debnath 2008, 2009). In this respect, application of TDZ alone (Debnath, 2006; Zakaria *et al.*, 2014) or in combination with 2,4-D (Passey *et al.*, 2003; Cappelletti *et al.*, 2016) or with IBA (Yonghua *et al.*, 2005; Sönmez and Kafkas, 2012) were effective for adventitious shoot regeneration in strawberry. The variability in the regeneration rates obtained in the different studies is due to the use of different concentrations of auxin and cytokinin, various explant types and several strawberry cultivars, indicating that each explant type for each genotype has specific requirements of auxin and cytokinin in the medium which are vital for regeneration (Zakaria *et al.*, 2014).

Chitosan (ß-(1, 4)-glucosamine polymer) is one of the most common biodegradable polymers obtained from the deacetylation of chitin which is present in shells of crustaceans, cuticles of insects and the cell wall of fungi and some algae (Sanford, 2002). Chitosan has many applications in industry (waste water treatment, membrane technology, pulp and paper, and medical and cosmetics products) and in agriculture (fruit coating, growth stimulator, and stimulator for the immunity and resistance of plants against fungi and viruses) (Uthairatanakij *et al.*, 2007). In tissue culture, chitosan has widely been used as elicitor to stimulate secondary metabolites production (Putalun *et al.*, 2007). Moreover, chitosan has been reported to act as a plant growth stimulator in tissue culture of orchids (Nge *et al.*, 2006; Nahar *et al.*, 2011) and potato (Kowalski *et al.*, 2006; Asghari-Zakaria *et al.*, 2009). To my knowledge, there are no reports on the effect of chitosan on the regeneration of strawberry.

This study aimed to establish and optimize an efficient regeneration system for strawberry cv. Festival from different types of explant (leaf discs, petioles, stipules, and roots) *via* studying the effect of different concentrations of IBA (0.2 or 0.5 mg $l^{-1}$) with TDZ (1.0, 1.5 or 2.0 mg $l^{-1}$) or BA (1.0, 2.0 or 3.0 mg $l^{-1}$) and supplemented with different concentrations of chitosan (0, 25 or 50 mg $l^{-1}$). In addition, the effect of chitosan at different concentrations on shoot multiplication was tested.

## Materials and Methods

The present study was conducted during 2014 to establish and optimize an efficient regeneration system for strawberry cv. Festival from different types of explant cultured on media

containing different concentrations of IBA with TDZ or BA and supplemented with different concentrations of chitosan.

*Establishment of Strawberry cv. Festival in vitro*

Strawberry cv. Festival was established *in vitro* using runner tips. Runners with 2-3 unfolded leaves were excised from screenhouse grown plants and washed under running tap water for 5 min to remove surface contaminants. Under the aseptic conditions of the laminar air-flow cabinet, the runners were sterilized by immersion in 1% sodium hypochlorite solution (with five drops of Tween20 per liter) for 20 min and then washed four times with sterile distilled water, 5 min every wash. The runner tips (1-2 mm) were dissected under a binocular microscope and cultured on strawberry initiation medium (López-Aranda *et al.*, 1994). The medium consisted in the macroelements of the $N_{30}K$ mineral formulation (Margara, 1986), MS microelements and vitamins (Murashige and Skoog, 1962), 100 mg/l myo-inositol, 2% sucrose, 4.92 µM IBA, 0.44 µM BA and 0.29 µM $GA_3$. The medium pH was adjusted to 5.74, solidified with 0.7% Phyto agar (Duchefa), and later autoclaved for 4 min at 121 ºC and 0.1 MPa pressure to dissolve the agar. Then, the medium was distributed into 25x150 mm test tubes with a volume of 10 ml of medium and autoclaved for 20 min at 121 ºC and 0.1 MPa pressure.

The *in vitro* cultures were maintained in a growth chamber at 25 ±2 °C under 16/8 h (day/night) photoperiod, with 40 µmol $m^{-2}$ $s^{-1}$ irradiance provided by fluorescent lamps. Shoots derived from runner tips were subcultured at 8-week intervals in 500-ml glass jars containing 150 ml of López-Aranda *et al.* (1994) micropropagation medium modified by Barceló *et al.* (1998) ($N_{30}K$ macroelements, MS vitamins, 2% sucrose and 2.21 µM kinetin) to provide enough material to initiate the regeneration experiment.

*Regeneration experiment*

The adventitious shoot regeneration capability of strawberry cv. Festival using different kind of explants was tested. Explants were taken from shoots proliferating in the modified micropropagation medium of López-Aranda *et al.* (1994), at 3-4 weeks from the last subculture. Leaf discs, petioles, stipules, and roots were used as explants. Leaf discs were cut from newly expanded leaves with a cork borer giving discs of 5x5 mm. All leaf explants were cultured with the adaxial surface in contact with the regeneration medium used. Petioles and roots were cut with a scalpel into 2-cm explants and cultured horizontally. Stipules were also cultured horizontally.

The shoot regeneration medium used was that contained the macroelements of the $N_{30}K$ mineral formulation (Margara, 1986), with MS microelements, myo-inositol and vitamins (Murashige and Skoog, 1962) supplemented with different concentrations of IBA (0.2 or 0.5 mg $l^{-1}$) with TDZ (1.0, 1.5 or 2.0 mg $l^{-1}$) or BA (1.0, 2.0 or 3.0 mg $l^{-1}$). Combinations of the auxin and cytokinins in the regeneration media were based on those previously used in other studies. In all cases, media were supplemented with 2% sucrose and Phyto-agar (0.7%) was added and the pH was adjusted to 5.74. The medium was autoclaved for 4 min at 121 ºC and 0.1 MPa pressure to dissolve the agar. Then, the medium was distributed into 175–ml glass jar containing 35 ml and autoclaved for 20 min at 121 ºC and 0.1 MPa pressure. Various concentrations of filter-sterilized (0.22 µm filter) chitosan (0, 25 or 50 mg/l) were added. After an initial dark treatment of two weeks, all cultures were placed at 25 ± 2˚C with a daily photoperiod of 16 h light provided by Sylvania Gro-lux lamps (40 µmol $m^{-2}$ $s^{-1}$ irradiance level).

*Multiplication experiment*

The shoots regenerated derived from leaf discs from the regeneration experiments were multiplicated in 500-ml glass jars containing 150 of López-Aranda *et al.* (1994) micropropagation medium modified by Barceló *et al.* (1998) ($N_{30}K$ macroelements, MS vitamins, 2% sucrose and 2.21 µM kinetin) and supplemented with the above-mentioned concentrations of chitosan. All cultures

were placed at 25 ± 2˚C with a daily photoperiod of 16 h light provided by Sylvania Gro-lux lamps (40 µmol $m^{-2}$ $s^{-1}$ irradiance level).

*Experimental design, data recorded and statistical analysis*

In the regeneration experiment, twenty explants in each of three replicates per treatment were used, at a density of ten explants per jar. Completely randomized design was performed. Data were taken throughout 3 recultures at four-week intervals. The regeneration rate was calculated as the percentage of cultured explants that produced shoots. Number of shoots/ explant was also calculated. Data are shown as means ± standard error of 60 explants per treatment.

In the multiplication experiment, ten shoots in each of three replicates per treatment were used, at a density of two shoots per jar. Completely randomized design was performed. Data were taken throughout 3 subcultures at four-week intervals. Number of plantlets/shoot was recorded. All data of number of plantlets/shoot were subjected to an analysis of variance using the CoStat package program (version 6.303; CoHort Software, USA). The differences among these means were compared using the least significance difference (LSD) at $p \leq 0.05$.

**Results**

Strawberry cv. Festival used in this study was recently introduced and currently grown in Egypt. In addition, the explant types used were chosen on the basis of being the most used in the previous studies for other strawberry cultivars. Various combinations of auxin (IBA) and cytokinin (TDZ or BA) in the regeneration media were tested to determine their effects on the regeneration efficiencies from a range of explant types for Festival cultivar under different concentrations of chitosan.

**Adventitious shoot regeneration from leaf discs**

The obtained data revealed that leaf discs proved to be a good source of explant material for regeneration of Festival cultivar. The regeneration efficiencies of leaf discs varied from 58 to 100% (Table 1). A medium containing 0.5 mg $l^{-1}$ IBA and 1.5 mg $l^{-1}$ TDZ supplemented with 25 mg $l^{-1}$ chitosan was found to be the most efficient combination which gave the highest level of regeneration (100%). On the contrary, the combination of 0.2 mg $l^{-1}$ IBA and 1.0 mg $l^{-1}$ BA without adding chitosan gave the lowest value of regeneration efficiency (58%). Data in Table 1 also showed that generally media containing high concentration of IBA with moderate concentrations of both cytokinins were the suitable combinations for obtaining the highest levels of regeneration form leaf discs. In addition, TDZ was found to be more effective than BA. In all media, chitosan adding at 25 mg $l^{-1}$ improved the shoot regeneration percentages.

Concerning the number of shoots regenerated per explant, media containing 0.5 mg $l^{-1}$ IBA and 1.5 or 2.0 mg $l^{-1}$ TDZ gave the highest number of shoots/leaf disc, whereas media containing low concentrations of IBA and BA gave the lowest values. Again, application of chitosan enhanced the regenerated shoots/explant. A combination of 0.5 mg $l^{-1}$ IBA, 1.5 mg $l^{-1}$ TDZ, and 25 mg $l^{-1}$ chitosan gave the highest number of shoots (Table 1).

**Adventitious shoot regeneration from petioles**

Data in Table 2 showed that the regeneration efficiencies of petioles varied from 0 to 40%. A medium containing 0.5 mg $l^{-1}$ IBA and 1.5 mg $l^{-1}$ TDZ supplemented with 25 mg $l^{-1}$ chitosan was found to be the most efficient combination which gave the highest level of regeneration, while the media containing low concentration of auxin (0.2 mg $l^{-1}$ IBA) with low concentrations of both cytokinins (1.0 mg $l^{-1}$ TDZ or 1.0 mg $l^{-1}$ BA) without chitosan addition did not demonstrate any regeneration. Anew, TDZ was found to be more effective than BA especially under high concentration of IBA.

**Table 1.** Effect of auxin and cytokinin combinations and different concentrations of chitosan on shoot regeneration percentages and number of shoots derived from leaf discs of strawberry cv. Festival. Data are means ± standard error of 60 explants per treatment (20 explants/replicate).

| Auxin | Cytokinin | Chitosan | | |
|---|---|---|---|---|
| | | 0.0 mg/l | 25 mg/l | 50 mg/l |
| Shoot regeneration percentages | | | | |
| IBA 0.2 mg/l | TDZ 1.0 mg/l | 59.00±2.9 | 68.33±1.7 | 66.66±3.3 |
| | TDZ 1.5 mg/l | 71.66±1.7 | 76.66±3.3 | 68.33±1.7 |
| | TDZ 2.0 mg/l | 65.33±4.4 | 71.66±1.7 | 68.33±1.7 |
| | BA 1.0 mg/l | 58.00±2.9 | 61.66±1.7 | 65.00±2.9 |
| | BA 2.0 mg/l | 66.66±1.7 | 70.00±2.9 | 63.33±1.7 |
| | BA 3.0 mg/l | 61.66±1.7 | 68.33±1.7 | 68.33±1.7 |
| IBA 0.5 mg/l | TDZ 1.0 mg/l | 75.00±2.9 | 83.33±1.7 | 76.66±3.3 |
| | TDZ 1.5 mg/l | 86.66±1.7 | 100.00±3.3 | 78.33±1.7 |
| | TDZ 2.0 mg/l | 59.66±1.7 | 86.66±3.3 | 68.33±1.7 |
| | BA 1.0 mg/l | 65.00±2.9 | 73.33±1.7 | 66.66±3.3 |
| | BA 2.0 mg/l | 76.66±1.7 | 86.66±3.3 | 68.33±1.7 |
| | BA 4.0 mg/l | 76.66±1.7 | 76.66±3.3 | 66.33±1.7 |
| Number of shoot regenerated/explant | | | | |
| IBA 0.2 mg/l | TDZ 1.0 mg/l | 2.51±0.03 | 2.71±0.01 | 2.93±0.01 |
| | TDZ 1.5 mg/l | 2.09±0.02 | 3.30±0.01 | 3.22±0.01 |
| | TDZ 2.0 mg/l | 1.80±0.02 | 2.50±0.03 | 1.93±0.00 |
| | BA 1.0 mg/l | 1.31±0.02 | 1.51±0.01 | 1.69±0.01 |
| | BA 2.0 mg/l | 1.92±0.01 | 1.88±0.05 | 1.68±0.01 |
| | BA 3.0 mg/l | 1.81±0.02 | 1.93±0.01 | 1.80±0.03 |
| IBA 0.5 mg/l | TDZ 1.0 mg/l | 2.43±0.04 | 2.98±0.02 | 2.80±0.02 |
| | TDZ 1.5 mg/l | 4.00±0.01 | 4.95±0.26 | 4.13±0.01 |
| | TDZ 2.0 mg/l | 3.72±0.02 | 4.05±0.11 | 3.78±0.02 |
| | BA 1.0 mg/l | 2.20±0.03 | 2.41±0.02 | 2.58±0.01 |
| | BA 2.0 mg/l | 2.80±0.01 | 2.79±0.04 | 2.56±0.03 |
| | BA 4.0 mg/l | 2.72±0.04 | 2.80±0.02 | 2.69±0.03 |

**Table 2.** Effect of auxin and cytokinin combinations and different concentrations of chitosan on shoot regeneration percentages and number of shoots derived from petioles of strawberry cv. Festival. Data are means ± standard error of 60 explants per treatment (20 explants/replicate).

| Auxin | Cytokinin | Chitosan | | |
|---|---|---|---|---|
| | | 0.0 mg/l | 25 mg/l | 50 mg/l |
| Shoot regeneration percentages | | | | |
| IBA 0.2 mg/l | TDZ 1.0 mg/l | 0.00±0.0 | 11.33±1.7 | 8.33±1.7 |
| | TDZ 1.5 mg/l | 15.00±2.9 | 23.33±1.7 | 18.33±1.7 |
| | TDZ 2.0 mg/l | 8.33±2.9 | 21.66±2.9 | 16.66±3.3 |
| | BA 1.0 mg/l | 0.00±0.0 | 11.66±1.7 | 13.33±1.7 |
| | BA 2.0 mg/l | 16.66±1.7 | 18.33±1.7 | 13.33±1.7 |
| | BA 3.0 mg/l | 11.66±1.7 | 18.33±1.7 | 18.33±1.7 |
| IBA 0.5 mg/l | TDZ 1.0 mg/l | 23.33±3.33 | 31.66±1.7 | 25.00±2.9 |
| | TDZ 1.5 mg/l | 33.33±1.7 | 40.00±2.9 | 20.00±2.9 |
| | TDZ 2.0 mg/l | 23.33±1.7 | 31.66±3.3 | 18.33±1.7 |
| | BA 1.0 mg/l | 11.66±1.7 | 20.00±2.9 | 13.33±1.7 |
| | BA 2.0 mg/l | 25.00±2.9 | 28.33±1.7 | 16.66±1.7 |
| | BA 4.0 mg/l | 13.33±1.7 | 23.33±4.4 | 8.33±1.7 |
| Number of shoot regenerated/explant | | | | |
| IBA 0.2 mg/l | TDZ 1.0 mg/l | 0.00±0.0 | 1.00±0.02 | 1.00±0.03 |
| | TDZ 1.5 mg/l | 1.18±0.03 | 1.36±0.03 | 1.33±0.02 |
| | TDZ 2.0 mg/l | 1.75±0.05 | 1.95±0.02 | 1.95±0.01 |
| | BA 1.0 mg/l | 0.00±0.0 | 1.42±0.04 | 1.57±0.02 |
| | BA 2.0 mg/l | 1.00±0.02 | 1.18±0.03 | 1.24±0.04 |
| | BA 3.0 mg/l | 0.95±0.03 | 1.02±0.03 | 1.00±0.03 |
| IBA 0.5 mg/l | TDZ 1.0 mg/l | 2.15±0.03 | 2.66±0.01 | 2.65±0.03 |
| | TDZ 1.5 mg/l | 2.35±0.02 | 3.00±0.05 | 3.10±0.04 |
| | TDZ 2.0 mg/l | 2.49±0.01 | 3.11±0.09 | 3.46±0.03 |
| | BA 1.0 mg/l | 1.90±0.02 | 2.33±0.04 | 2.41±0.02 |
| | BA 2.0 mg/l | 1.75±0.02 | 2.60±0.03 | 2.62±0.04 |
| | BA 4.0 mg/l | 1.70±0.03 | 2.59±0.01 | 2.61±0.08 |

Concerning the number of shoots regenerated per explant, Data in Table 2 also revealed that a medium containing 0.5 mg $l^{-1}$ IBA and 2.0 mg $l^{-1}$ TDZ supplemented with 50 mg $l^{-1}$ chitosan gave the highest number of shoots/petiole, whereas the media containing low concentrations of IBA and TDZ or BA without chitosan supplementation did not produce any shoots.

**Adventitious shoot regeneration from stipules**

Stipules exhibited regeneration efficiencies ranging from 8.33 to 47.66% (Table 3). A medium containing 0.5 mg $l^{-1}$ IBA and 1.5 mg $l^{-1}$ TDZ supplemented with 25 mg $l^{-1}$ chitosan gave the highest level of regeneration from stipules. On the contrary, a combination of 0.2 mg $l^{-1}$ IBA and 1.0 mg $l^{-1}$ BA without chitosan gave the lowest value of regeneration efficiency. Once again, it is observed that effectiveness of TDZ over BA.

As for the number of shoots regenerated per explant, a medium containing 0.5 mg $l^{-1}$ IBA and 2.0 mg $l^{-1}$ TDZ supplemented with 50 mg $l^{-1}$ chitosan produced the highest number of shoots/stipule, while the media containing 0.2 mg $l^{-1}$ IBA and BA without chitosan adding gave the lowest numbers of shoots (Table 3).

**Table 3.** Effect of auxin and cytokinin combinations and different concentrations of chitosan on shoot regeneration percentages and number of shoots derived from stipules of strawberry cv. Festival. Data are means ± standard error of 60 explants per treatment (20 explants/replicate).

| Auxin | Cytokinin | Chitosan | | |
|---|---|---|---|---|
| | | 0.0 mg/l | 25 mg/l | 50 mg/l |
| Shoot regeneration percentages | | | | |
| IBA 0.2 mg/l | TDZ 1.0 mg/l | 15.00±2.9 | 18.33±1.7 | 16.66±3.3 |
| | TDZ 1.5 mg/l | 21.66±2.9 | 25.66±3.3 | 19.33±1.7 |
| | TDZ 2.0 mg/l | 11.00±1.7 | 20.66±2.9 | 19.33±1.7 |
| | BA 1.0 mg/l | 8.33±4.4 | 11.66±1.7 | 16.00±2.9 |
| | BA 2.0 mg/l | 15.66±1.7 | 19.00±1.7 | 13.00±1.7 |
| | BA 3.0 mg/l | 11.66±1.7 | 18.00±2.9 | 18.33±1.7 |
| IBA 0.5 mg/l | TDZ 1.0 mg/l | 23.00±2.9 | 32.33±1.7 | 25.66±3.3 |
| | TDZ 1.5 mg/l | 39.66±1.7 | 47.66±3.3 | 26.33±1.7 |
| | TDZ 2.0 mg/l | 25.66±1.7 | 39.66±3.3 | 25.33±1.7 |
| | BA 1.0 mg/l | 14.00±1.7 | 22.33±1.7 | 15.00±3.3 |
| | BA 2.0 mg/l | 24.66±1.7 | 37.66±2.9 | 17.66±1.7 |
| | BA 4.0 mg/l | 16.33±1.7 | 28.00±3.3 | 10.00±1.7 |
| Number of shoot regenerated/explant | | | | |
| IBA 0.2 mg/l | TDZ 1.0 mg/l | 1.25±0.04 | 1.43±0.04 | 1.55±0.04 |
| | TDZ 1.5 mg/l | 1.40±0.06 | 1.62±0.05 | 1.68±0.07 |
| | TDZ 2.0 mg/l | 1.85±0.09 | 1.95±0.01 | 2.00±0.03 |
| | BA 1.0 mg/l | 1.00±0.03 | 1.28±0.06 | 1.36±0.04 |
| | BA 2.0 mg/l | 1.00±0.07 | 1.36±0.03 | 1.47±0.08 |
| | BA 3.0 mg/l | 1.00±0.06 | 1.12±0.05 | 1.10±0.06 |
| IBA 0.5 mg/l | TDZ 1.0 mg/l | 2.65±0.08 | 2.88±0.07 | 3.00±0.04 |
| | TDZ 1.5 mg/l | 2.73±0.05 | 3.35±0.05 | 3.55±0.07 |
| | TDZ 2.0 mg/l | 2.80±0.04 | 3.59±0.13 | 3.75±0.03 |
| | BA 1.0 mg/l | 2.00±0.06 | 2.49±0.05 | 2.55±0.03 |
| | BA 2.0 mg/l | 2.25±0.04 | 2.75±0.02 | 2.50±0.01 |
| | BA 4.0 mg/l | 2.20±0.09 | 2.50±0.02 | 2.25±0.02 |

**Adventitious shoot regeneration from roots**

Data in Table 4 clearly demonstrated that the regeneration efficiencies of roots varied from 11 to 57.66%. A combination of 0.2 mg $l^{-1}$ IBA and 1.0 mg $l^{-1}$ BA without adding chitosan gave the lowest value of regeneration efficiency, while the combination of 0.5 mg $l^{-1}$ IBA and 2.0 mg $l^{-1}$ TDZ supplemented with 25 mg $l^{-1}$ chitosan gave the highest one. Data also showed the effectiveness of TDZ on regeneration efficiency.

Data in Table 4 also indicated that a medium containing 0.5 mg $l^{-1}$ IBA and 2.0 mg $l^{-1}$ TDZ supplemented with 25 mg $l^{-1}$ chitosan produced the highest number of shoots/root. On the contrary, the combination of 0.2 mg $l^{-1}$ IBA and 1.0 mg $l^{-1}$ BA without chitosan addition gave the lowest number of shoots.

**Table 4.** Effect of auxin and cytokinin combinations and different concentrations of chitosan on shoot regeneration percentages and number of shoots derived from roots of strawberry cv. Festival. Data are means ± standard error of 60 explants per treatment (20 explants/replicate).

| Auxin | Cytokinin | Chitosan | | |
|---|---|---|---|---|
| | | 0.0 mg/l | 25 mg/l | 50 mg/l |
| Shoot regeneration percentages | | | | |
| IBA 0.2 mg/l | TDZ 1.0 mg/l | 25.00±1.7 | 28.33±1.7 | 26.66±2.9 |
| | TDZ 1.5 mg/l | 18.66±1.7 | 31.66±3.3 | 28.33±1.7 |
| | TDZ 2.0 mg/l | 33.33±1.7 | 36.66±1.7 | 28.33±1.7 |
| | BA 1.0 mg/l | 11.00±2.9 | 17.66±1.7 | 20.00±2.9 |
| | BA 2.0 mg/l | 26.66±1.7 | 28.00±3.3 | 23.33±1.7 |
| | BA 3.0 mg/l | 25.66±1.7 | 28.33±3.3 | 24.33±2.9 |
| IBA 0.5 mg/l | TDZ 1.0 mg/l | 35.00±2.9 | 43.33±4.4 | 36.66±3.3 |
| | TDZ 1.5 mg/l | 46.66±1.7 | 56.66±3.3 | 47.33±1.7 |
| | TDZ 2.0 mg/l | 42.66±1.7 | 57.66±1.7 | 49.33±3.3 |
| | BA 1.0 mg/l | 25.00±2.9 | 33.33±1.7 | 26.66±3.3 |
| | BA 2.0 mg/l | 35.66±4.4 | 44.66±3.3 | 40.33±1.7 |
| | BA 4.0 mg/l | 24.66±3.3 | 34.66±1.7 | 19.33±2.9 |
| Number of shoot regenerated/explant | | | | |
| IBA 0.2 mg/l | TDZ 1.0 mg/l | 1.35±0.01 | 1.12±0.02 | 1.25±0.02 |
| | TDZ 1.5 mg/l | 1.35±0.03 | 2.00±0.04 | 2.00±0.02 |
| | TDZ 2.0 mg/l | 1.55±0.01 | 2.15±0.01 | 2.00±0.03 |
| | BA 1.0 mg/l | 1.00±0.01 | 1.62±0.03 | 1.63±0.03 |
| | BA 2.0 mg/l | 1.66±0.04 | 1.84±0.05 | 1.77±0.02 |
| | BA 3.0 mg/l | 1.85±0.02 | 1.99±0.01 | 1.89±0.03 |
| IBA 0.5 mg/l | TDZ 1.0 mg/l | 2.65±0.04 | 3.00±0.05 | 3.01±0.03 |
| | TDZ 1.5 mg/l | 2.88±0.01 | 3.41±0.02 | 3.33±0.04 |
| | TDZ 2.0 mg/l | 2.80±0.03 | 3.45±0.08 | 3.22±0.07 |
| | BA 1.0 mg/l | 2.15±0.05 | 2.51±0.03 | 2.50±0.04 |
| | BA 2.0 mg/l | 2.74±0.03 | 2.95±0.02 | 2.94±0.05 |
| | BA 4.0 mg/l | 2.69±0.07 | 2.70±0.04 | 2.69±0.05 |

**Effect of chitosan on shoot multiplication**

The effect of chitosan application at different concentrations on shoot multiplication regenerated from leaf discs was investigated throughout 3 subcultures at four-week intervals. It is observed that chitosan application at 25 or 50 mg $l^{-1}$ accelerated the appearance of the proliferation rate by 5-7 days as compared to the control (without chitosan adding). Moreover, both concentrations of chitosan increased number of plantlets/explant without significant differences (Fig. 1).

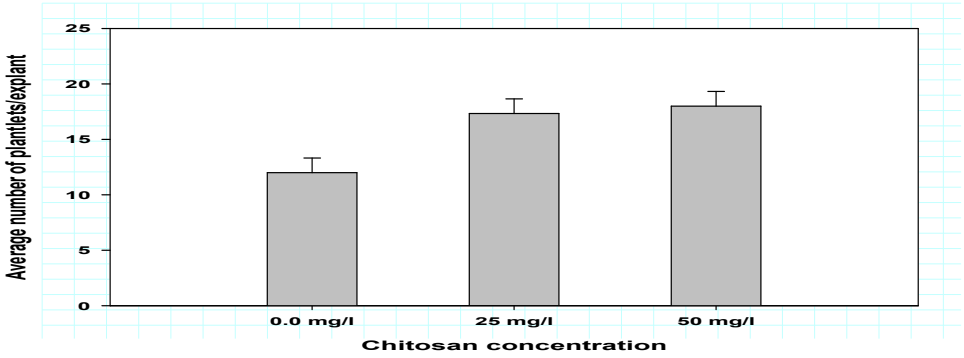


**Fig. 1:** Effect of chitosan concentrations on average number of plantlets/explant of strawberry cv. Festival.Vertical bars indicate the LSD value at $p \leq 0.05$.

## Discussion

As an initial and a prerequisite step towards the establishment of a protocol for genetic transformation or somaclonal variation of Festival cultivar, the shoot regeneration ability of different kinds of explants (leaf discs, petioles, stipules, and roots) *via* studying the influence of different concentrations of IBA (0.2 or 0.5 mg $l^{-1}$) with TDZ (1.0, 1.5 or 2.0 mg $l^{-1}$) or BA (1.0, 2.0 or 3.0 mg $l^{-1}$) and supplemented with different concentrations of chitosan (0, 25 or 50 mg $l^{-1}$). Furthermore, the effects of chitosan at different concentrations on shoot multiplication have been tested.

Four types of explants (leaf discs, petioles, stipules, and roots) were tested for regeneration efficiency of Festival. In previous studies, leaf discs (Liu and Sanford, 1988; Nehra *et al.*, 1989; Sorvari *et al.*, 1993; Damiano *et al.*, 1997; Barceló *et al.*, 1998; Flores *et al.*, 1998; Passey *et al.*, 2003; Qin *et al.*, 2005; Debnath, 2006; Folta *et al.*, 2006; Husaini and Srivastava, 2006; Husaini and Abdin, 2007; Ara *et al.*, 2012; Mahmoud and Kosar, 2013; Nasri and Bahramnejad, 2013; Cappelletti *et al.*, 2016), petioles (Rugini and Orlando, 1992; Damiano *et al.*, 1997; Passey *et al.*, 2003; Mezzetti *et al.*, 2004; Folta *et al.*, 2006), stipules (Rugini and Orlando, 1992; Damiano *et al.*, 1997; Monticelli *et al.*, 2002; Passey *et al.*, 2003; Sönmez and Kafkas, 2012), and roots (Rugini and Orlando, 1992; Passey *et al.*, 2003) were used for other strawberry cultivars. The obtained data revealed that leaf discs proved to be a good source of explant material for regeneration of Festival cultivar. In contrast, petioles exhibited the lowest rates of regeneration. Both stipules and roots exhibited intermediate values of regeneration frequencies, and were relatively similar. In accordance with these findings, earlier studies on strawberry regeneration showed that leaf discs had the highest regeneration capacity of all strawberry plant tissues tested (Liu and Sanford, 1988; Nehra *et al.*, 1989; Jelenkovic *et al.*, 1990; Popescu *et al.*, 1997; Passey *et al.*, 2003; Ara *et al.*, 2012; Mahmoud and Kosar, 2013). The differences in regeneration capacities from different types of explants may be due to the variation in the endogenous levels of the growth hormones in these explants.

The combination of auxins and cytokinins is another key factor regulating the adventitious shoot regeneration, and the concentration ratio between these plant growth regulators is critical to determine specific organogenesis processes from different explants (Su *et al.*, 2011). In this study, one type of auxin (IBA) and two types of cytokinins (BA or TDZ) at different concentrations were tested for adventitious shoot regeneration from different explants. In this sense, the combination of IBA and BA was most commonly used for strawberry regeneration (Sorvari *et al.*, 1993; Barceló *et al.*, 1998; Husaini and Srivastava, 2006). In addition, thidiazuron (TDZ) or 1-phenyl-3-(1,2,3-thiadiazol-5-yl) urea, a synthetic phenylurea-type cytokinin, has widely been used to promote shoot proliferation and regeneration in strawberry (Sutter *et al.*, 1997; Flores *et al.*, 1998; Murthy *et al.*, 1998; Barceló *et al.*, 1998; Schaart *et al.*, 2002; Passey *et al.*, 2003; Zhao *et al.*, 2004; Qin *et al.*, 2005; Yonghua *et al.*, 2005; Oosumi *et al.*, 2006; Landi and Mezzetti, 2006; Cappelletti *et al.*, 2016), and can substitute the use of BA. Furthermore, TDZ possesses both cytokinin and auxin-like properties (Husaini and Abdin, 2007). The obtained data revealed that IBA combined with TDZ gave the highest regeneration frequency and improved the number of shoots for all tested explants. Similarly, previous studies demonstrated that TDZ in combination with IBA was effective for shoot regeneration in some other strawberry cultivars (Qin *et al.*, 2005; Hanhineva *et al.*, 2005; Yonghua *et al.*, 2005). In addition, the obtained results demonstrated that TDZ was more effective than BA for shoot regeneration from different types of explants. These results are in agreement with some investigators who found that TDZ was more effective than BA for callus growth and shoot regeneration in strawberry (Flores *et al.*, 1998). However, Wang *et al.* (2003) found that the inclusion of TDZ in the shoot regeneration medium inhibited root formation in obtained shoots, making BA rather than TDZ the optimum hormone for shoot regeneration. In this work, all shoots obtained and all the shoots were successfully rooted (data not shown). In spite of these findings, neither the basic mechanism of TDZ action nor the molecular action is understood. However, various reports indicated that TDZ may act through modulation of the endogenous plant growth regulators, either directly or as a result of induced stress (Taiz *et al.*, 2014). In this connection, Hare and Van Staden (1994) stated that the application of TDZ in tissue culture medium increased the internal cytokinin levels and the reduction of cytokinin oxidase activation. Also, it is found that TDZ involved in the synthesis of auxin by increasing the levels of

IAA and its precursor tryptophan, and also involved in the modification of cell membranes, energy levels, nutrient uptake, or nutrient assimilation (Murthy *et al.*, 1998).

Chitosan, a biodegradable polymer, has widely been used as a plant growth stimulator in tissue culture of some plant species like orchids (Nge *et al.*, 2006; Nahar *et al.*, 2011) and potato (Kowalski *et al.*, 2006; Asghari-Zakaria *et al.*, 2009). The obtained results revealed that chitosan adding at 25 mg $l^{-1}$ enhanced the regeneration rates of all tested explants and also improved number of shoots regenerated. Similarly, the enhancing effect of chitosan on *in vitro* shoot and plantlet regeneration has been observed in lily (Kanchanapoom *et al.*, 2012), apple (Dastjerd *et al.*, 2013), and spring rapeseed (Ahmadi and Shariatpanahi, 2015). Moreover, the influence of different concentrations of chitosan on shoot multiplication was investigated. The obtained results indicated that chitosan application increased number of plantlets/explant. In spite of the chitosan stimulations, its exact mode of action is still unknown. However, Uthairatanakij *et al.* (2007) found that chitosan enhanced growth and development by auxin biosynthesis pathway via tryptophan-independent pathway.

## Conclusion

Efficient adventitious shoot regeneration was optimized for Festival cultivar using leaf discs, petiole, stipules, and roots *via* application of various combinations of the auxin (IBA) and cytokinin (TDZ or BA) and chitosan application. TDZ was more effective than BA and chitosan application acted as a growth stimulator for shoot regeneration from all tested explants. The medium containing 0.5 mg $l^{-1}$ IBA and 1.5 mg $l^{-1}$ TDZ supplemented with 25 mg $l^{-1}$ chitosan was found to be the most efficient combination which gave the highest level of regeneration from leaf disc (100%).

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