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FRUITS OF MICROPROPAGATED STRAWBERRY (*FRAGARIAANANASSA*) PLANTS EXHIBITED HIGHER ANTIOXIDANT METABOLITES AS COMPARED TO *IN VIVO* GROWN PLANTS

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Abstract

Berry crops have the highest concentrations of antioxidant metabolites, which have a considerable potential to reduce the risk of a variety of degenerative diseases. This work aimed to provide a standardized protocol for microporpagation of *Fragaria ananassa* from stem segment explants and to compare the antioxidant metabolites of aqueous extracts of fruits from *In vitro* and *In vivo* derived plants. Earliest callus induction (7.33 days) with maximum response (93.33%) was observed on MS medium containing 0.5 mg L^{-1} 2,4-D + 0.5 mg L^{-1}NAA. The earliest shoot induction (within 13.33 days from callus masses), maximum shooting frequency (80.33%) and highest number of shoots (a mean of 5.67 shoots per callus) was obtained on MS media supplemented with 1.5 mg L^{-1}TDZ. Earliest rooting (8.67 days), maximum rooting response (66.33%) and highest number of roots (6.33 per individual shoots) was noted on MS half strength media containing 1 mg L^{-1}IBA. Number of fruits and yield per plant was higher *In vivo* as compared to micropropagated plants. In contrast, total anthocyanin, total phenolic and ascorbic acid content were found higher in micropropagated plants as compared to *In vivo* plants. High performance liquid chromatography (HPLC) showed that the anthocyanins pigments from fruits of micro propagated plants were identical with field grown plants, and appeared on the ODS-column HPLC with higher retention time than the main pigments of field grown crop.These findings suggest that the fruits of micropropgated strawberry plants could be utilized as a source of bioactive substances with antioxidant properties in industrial applications.

Key words: Strawberry, Micropropagation, Plant growth regulators, Bioactive compounds.

Introduction

Strawberry (*Fragaria ananassa*) belongs to the family Rosacea, a cross-pollinated fruit crop and is monoecious. Strawberries are cultivated worldwide for their attractive and delicious fruits. Strawberry fruit is commonly liked for its red colour, sweetness, good aroma, and juicy texture. It is consumed either fresh or in processed foods as milkshakes, juice, jam, chocolates and ice cream. Strawberry flavors and aromas are also commonly used in products such as soap, perfume, lip gloss, and many others (Manganaris *et al*., 2014). In the 14th century French took the wild strawberry from the forest to their gardens for harvest. However, garden strawberry (*Fragaria x ananassa)* was first grown commercially in France, during the late 18th century. To date, twenty different species of strawberries are known and grown commercially in 70 countries (Gaafar & Samad, 2006).

Strawberry is an herbaceous perennial plant, genetically strong and can adapt to various climates**.** Strawberry plant anatomy is quite complex, they contain leaf, crown, stolen or runner, root system and daughter strawberry plant (Voth & Bringhurst, 1984).The crowns are the productive engines of a strawberry plant. From this, region both runners and flowering stalks are produced. As a result of runners root bud contact with soil and new strawberry plant clones are produced (Shulaev *et al*., 2011).

Strawberry fruits are berries in nature, soft in with pink and radish colours with many seeds surrounded in the surface and called achenes (Vallarino *et al.*, 2020). Strawberry fruits are a rich source of vitamins C and ellagic acid (polyphenol antioxidant). Its fruit contains bioactive compounds which are effective against several types of cancers (Basu *et al.*, 2014; Sakila *et al*., 2007). Strawberries are also a rich source of anthocyanin and possess high antioxidants which has many health benefits including prevents heart disorders (Jenkins *et al*., 2008) inhabits the growth of cancer (Simões *et al*., 2012) and anti-inflammatory in nature (Ram *et al*., 2013).

Strawberries are commercially propagated through runners; however, it is laborious, time-consuming, expensive and results in the transmission of viral infections (Torrico *et al.*, 2018). Plant tissue culture is a fascinating substitute technique to propagate strawberries around the year. Previous research showed that strawberry plants obtained through micropropagation remained high yielded (Karhu & Hakala, 2000; Kikas *et al*., 2006), disease-free (Mohan *et al*., 2005) and resistive against the soil pathogens (Biswas *et al*., 2007). Because it can generate multiple desirable new clones from a single source plant in a short period all year, micropropagation is an effective approach to improve existing wild berry fields as well as start a new farm (Debnath & Goyali, 2020).

Explant source, culturing media and plant growth regulators are the influential factors in micropropagation technology (Chukwujekwu *et al*., 2002). Stem segments and leaves of herbaceous plants are commonly used as explant sources for micropropagation purpose (Shen *et al.*, 2012).

Among the plant growth regulators Benzial amino purin (BAP), Napathelinacetic acid (NAA) and Indoleacetic acid (IAA) are commonly used in micropropagation of a variety of plant species (Nalawade *et al*., 2003). Thidiazuron (TDZ) has been identified as a potent plant growth regulator to enhance shoots proliferation in many plants species including wild mint (Faisal *et al*., 2014), Travancore Persian Violet *(*Kannan *et al.,* 2007*)* and oilseed rape (Biesaga-Kościelniak *et al*., 2010). Recently more researchers are interested in micropropagation because of its ability to improve biochemical properties in berry crops such as in blueberries(Goyali *et al*., 2015), cranberry (Debnath & An, 2019), lingonberry (Debnath and Arigundam, 2020). In addition, micropropagated berries have higher levels of phenolic compounds, which are a good source of antioxidants for the human body (Debnath & Goyali, 2020).

The current study's goals were (i) to propagate the stem segments of *F. ananassa* in controlled conditions, and (ii) to determine and compare the antioxidant metabolites potential of fruits from micropropagated plants with the fruits from *In vivo* plants.

Materials and Methods

Seed material and culture establishment: Seed of *Fragariaan anassa* 'Chandler' were obtained from the Institute of Fruit Science's germplasm bank, The University of Agriculture Peshawar Pakistan. Seeds were surface sterilized by washing with distilled water three times; followed by two minutes of treatment with 0.2 percent (w/v) $HgCl_2$. To eliminate any leftover $HgCl_2$, the seeds were rinsed in distilled water. MS basal media was supplemented with 2.5 percent sucrose (w/v) and 0.8 percent agar (w/v), and the pH was adjusted to 5.8 using 0.3 N HCL and NaOH solutions before autoclaving at 121°C for 20 minutes. Sterilized seeds were cultured in baby jars (5 seeds per jar) containing about 30 ml MS basal media. The cultures were kept in the dark for three days before being transferred to a culture chamber with a temperature of 25±2°C and relative humidity of 70%, a 16:8 h light/dark photoperiod, and cool white fluorescent lights for illumination.

Callus induction: Ex-plant source in the experiment were stem segments (5-7 mm length) derived from 10-11 days old *In vitro* produced seedlings. The excised stem segments were cultured on MS medium comprising varying levels of 2, 4-D alone or in combination with NAA (Fig. 1c). Explants were cultivated in Pyrex baby jars (5 x 9 cm) with 30 ml of culturing media. For each treatment 25 explants were cultured in 5 baby jars. Data on days to callus initiation and percent response of callus were recorded. The experiment was repeated three times to get 3 replicates (5 explant × 5 baby jars) for the concentration of each Plant growth regulator (PGR's) mixture. Cultures were maintained in a culture room at 25±2°C and 70% relative humidity, with a 16:8 h light/dark photoperiod and light supplied at an intensity of 60 µmol $m^{-2}s^{-1}$ by cool white fluorescent lamps.

Shoot regeneration: Established fresh calli were grown on MS media with varying amounts of TDZ alone and in combination with Kinetin to know the influence of plant growth regulators on shoot regeneration (Fig. 3). After 4 weeks of culture, data on days to shoot initiation, shooting frequency, and the number of shoots per callus were collected.

Roots induction from callus derived shoots: Regenerated shoots (4-5 cm long) were sub-cultured on freshly prepared medium comprising full and half strength of MS basal media fortified with varied amounts of IBA and NAA (Fig. 4). Data on days to root induction, root induction frequency and the number of roots were all kept track of.

Field trials: For fruiting, micropropagated and *In vivo* (runner-propagated) plants were grown in an open field. Weeds were eliminated from the soil and it was thoroughly prepared before planting. Healthy runner-propagated and micropropgated plants with a strong root system were chosen, the plants were planted in 1.50 cm long by 20 cm wide, 15–20 cm high raised beds. In a double row, twelve plants were planted (20 × 25 cm spacing) per bed. For each replication, 3 beds were having 2.7 m^2 area, total field area comprises 8.1 m^2 with total 108 experimental plants, other cultural practices were carried out in accordance with the guidelines set forth by Kikas *et al*., (2006).

Number of fruits and yield $plant^{-1}$: Every other day, the number of fruits per plant was counted on three randomly selected plants from each replication, and the total yield was represented in g $plant^{-1}$.

Analytical methods: Fresh pulp of fruits from micropropgated and field-grown plants were used for the determination of total phenolic content (TPC), total flavonoid content (TFC), Total Anthocyanin content and ascorbic acid content. The TPC and TFC of samples were determined according to the procedure of Irshad *et al*., (2018). Gallic acid (GA) was used as a reference for determining total phenolic content. With a UV–Visible spectrophotometer (Shimadzu-1650PC, Japan), the absorbance of extracts was compared to a Gallic acid reference curve (sigma; 0-100 mg ml^{-1}), and total phenolic content was reported as GA (Gallic acid equivalent) mg/100 g fresh weight of the pulp. For determination of TFC a standard curve of Rutin solution (sigma; 0-120 mg ml^{-1}) was drawn, the absorbance of fruit samples was detected by a spectrophotometer at 510 nm, and results were expressed as RE (Rutin equivalent) mg/100 g fresh weight of the pulp. Total anthocyanin was evaluated by the method of Ranganna, (1997). Briefly, 5 g of sample was blended in 10 ml HCL and ethanol solutions (85:15), by adding distilled water, the amount was increased to 100 ml, and the mixture was kept overnight before being filtered using Whatmanno.1 filter paper. After that, the filtrate's optical density (OD) was measured at 535 nm, and the formulas below were used to calculate total anthocyanin.

$$\text{Total OD/100g} = \frac{\text{OD} \times \text{volume make up} \times 100}{\text{Weight of sample}}$$

$$\text{Total anthocyanin (mg /100 g)} = \frac{\text{Total OD/100 g}}{98.2}$$

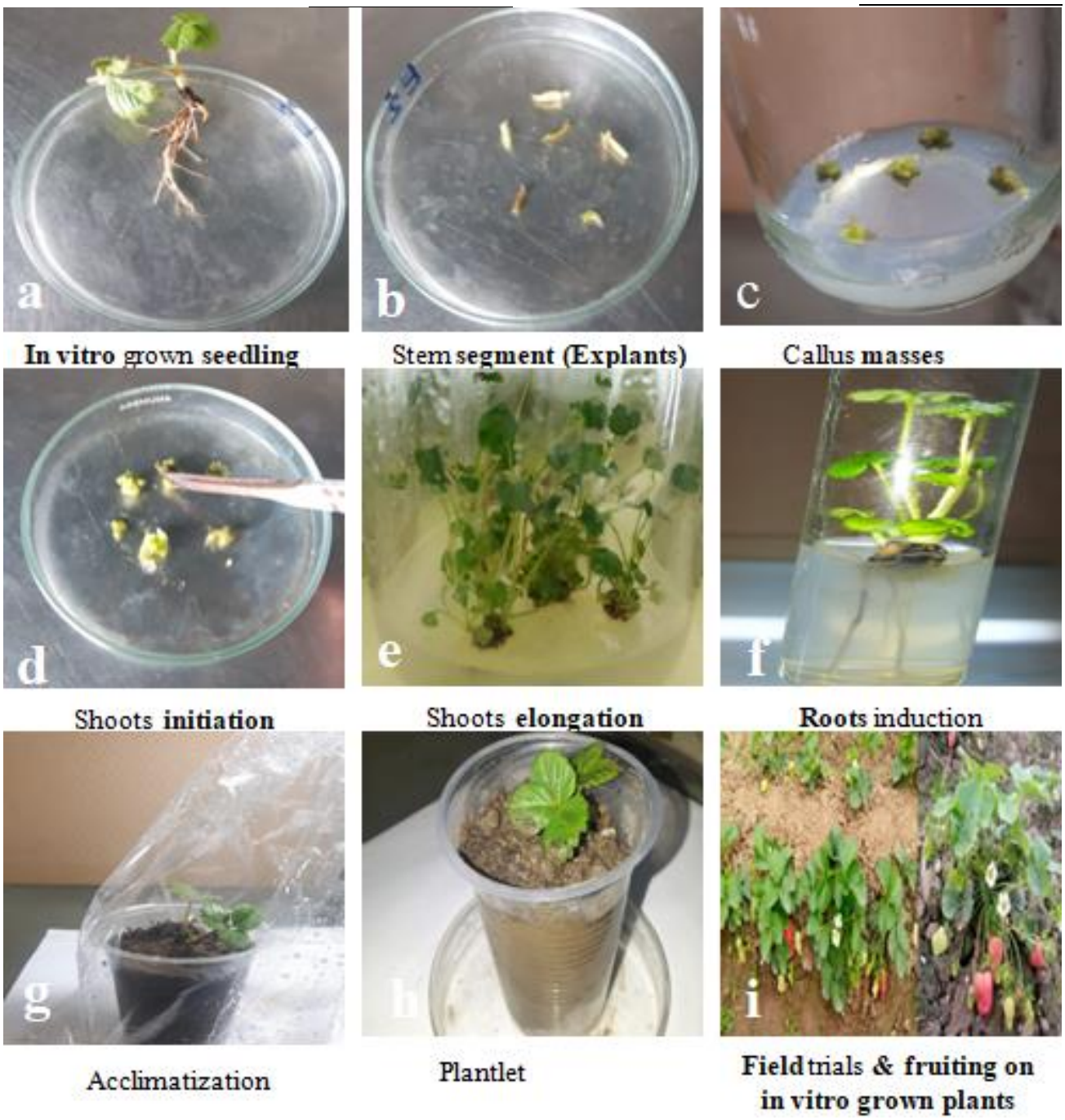

Fig. Micropropagation of Strawberry cv. Chandler (a) Twelve days old *In vitro* grown seedling (b) Stem segment explants (c) Callus induction (d & e) Shoots initiation and elongation (f) Roots induction (g & h) Acclimatization (i) Field trials and fruiting of *In vitro* grown plants.

HPLC analysis: Procedure, as described by Irshad *et al*., (2018), was adopted for individual anthocyanin detection and quantification. Fresh pulp from fruits of micropropagated and field-grown plants was taken (2 g), then using a mortar and pestle crushed into fine powder in liquid nitrogen. From each sample, 100 mg powder was taken, and incubated in 4 ml acidified methanol (methanol and 1% HCl, v/v) for 24 hours at 4°C. Using a chilled centrifuge (3K15; Sigma, Shanghai, China), the extracts were spun at 10,000 rpm for 10 minutes and filtered through 0.45 m filters. The samples wereanalysedon a series of 200 HPLC instruments (PerkinElmer Analytical Science, Shelton, USA) fitted with a diode array detection system and an AminexHPX-87H column (300 x 7.8 mm, Bio-Rad, USA), with the operating temperature set at 30°C. The mobile step for the anthocyanin profile was 7.5 percent (v/v) formic acid in acetonitrile (Solvent A) and water (Solvent B) at a flow rate of 1 mL min^{-1}, which was adapted from Simões *et al*., (2009) with only minor changes. The injection volume was set at 20 µL. The gradient began at 3% A and progressed to 15% A after 10 minutes, 25% A after 25 minutes, 40% A after 30 minutes, and 90% A after 45 minutes. Chromatograms were monitored at 225 nm from the diode array, and data were recorded between 200 and 600 nm.

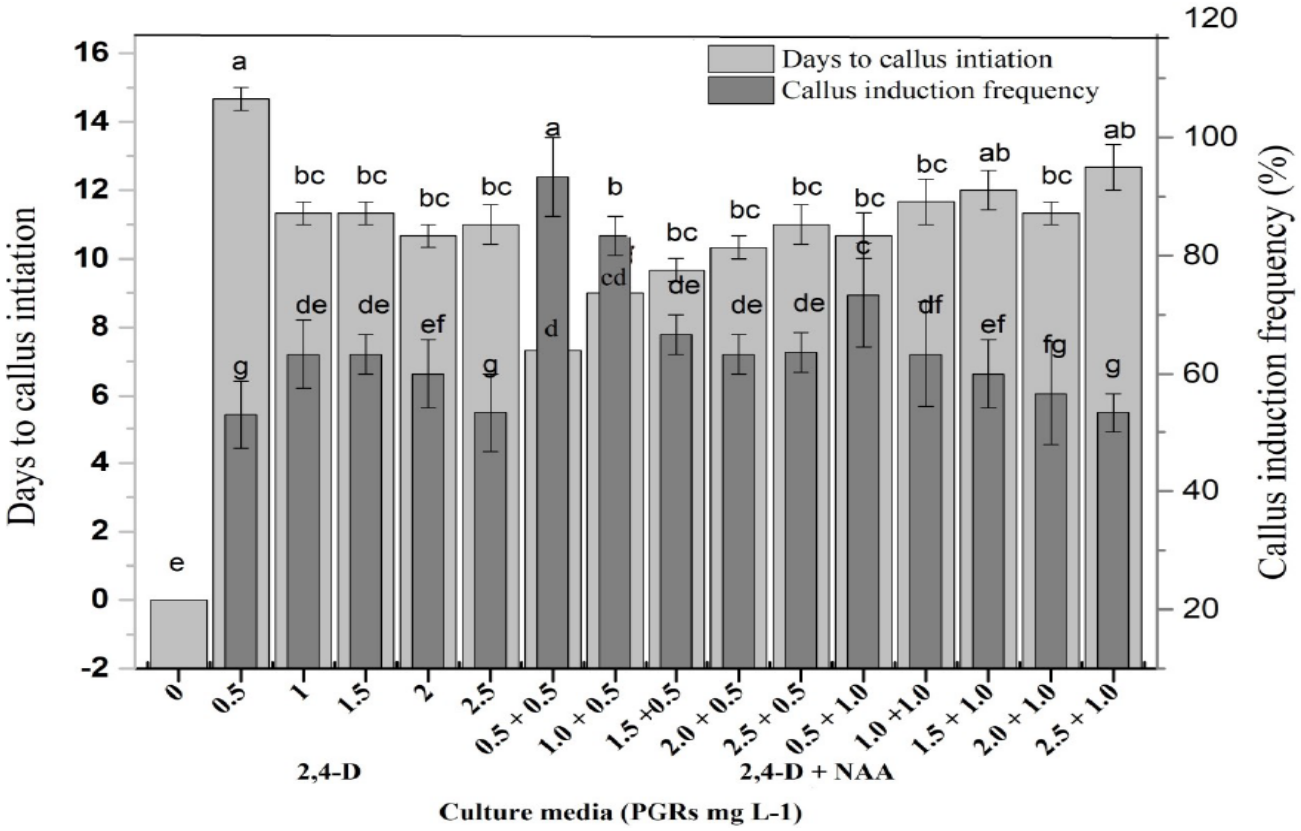

Fig. 2. Callus induction from stem segments of Strawberry cultured on16 different types of media, (2,4-D)2,4-Dichlorophenoxyacetic acid, (NAA) Naphthalene acetic acid, (PGRs) Plant growth regulators. Each bar represents mean ± SE; n = 3. Different letters above the bars in respective panels indicate significant differences between the treatments at $p<0.05$ (Tukey's test).

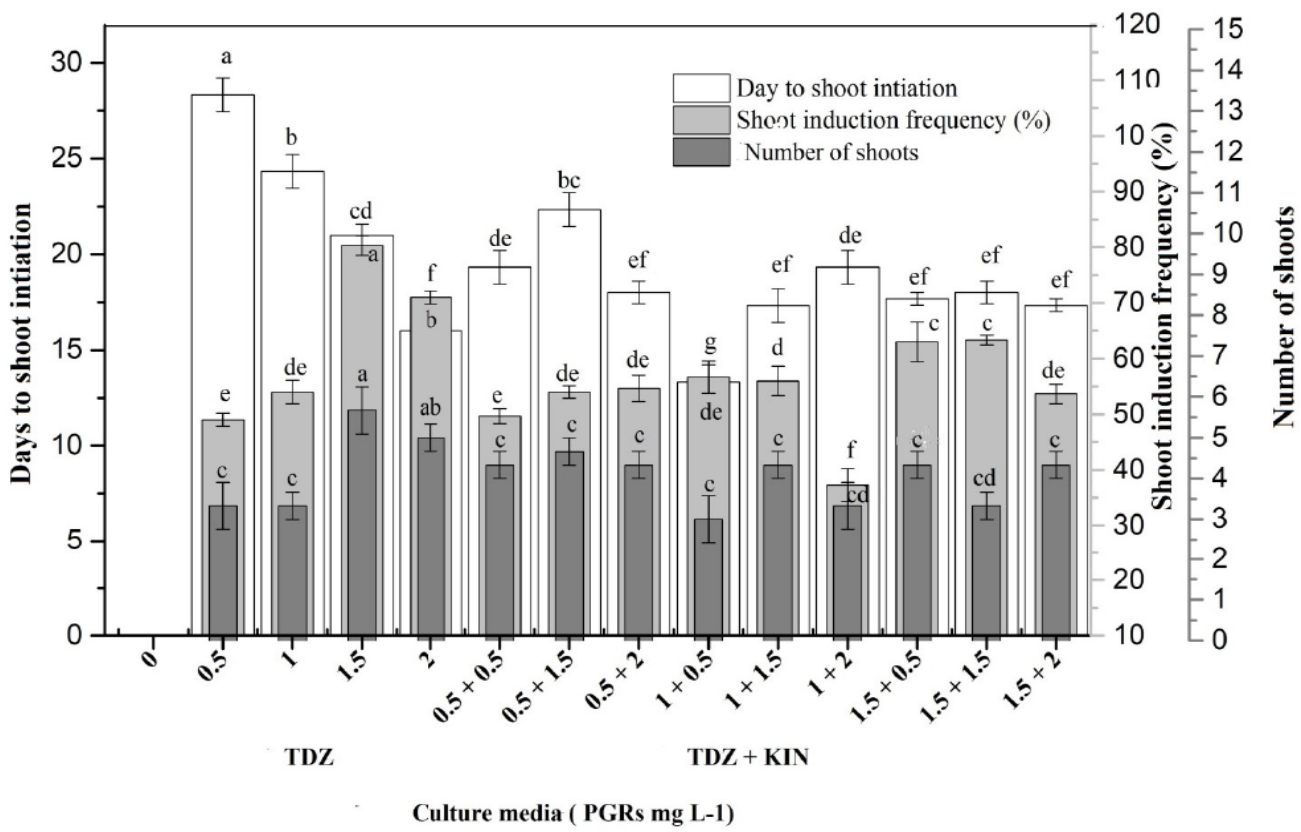

Fig. 3. Shooting from calluses grown on 14 different types of media, (TDZ) Thidiazuron, (KIN) Kinteen, (PGRs) Plant growth regulators. Each bar represents mean ± SE; n = 3. Different letters above the bars in respective panels indicate significant differences between the treatments at $p<0.05$ (Tukey's test).

Chemicals: Analytical grade chemicals supplied by Multi linkx enterprises, Solarbio (China), Sigma (U.S.A.) and E. Merck chemicals were used.

Statistical analysis

The experiments were conducted according to a completely randomized design, with each experiment being replicated three times. The control treatment was MS medium without plant growth regulators. All statistical analyses were conducted using one-way analysis of variance (ANOVA), and differences between treatment means were determined using the Least Significance Difference (LSD) test at a 5% level of probability, as recommended by Steel & Torrie (1986).

Results and Discussion

Days to callus initiation: After 7-10 days of culture on callus induction media, callus induction was observed (Fig. 1c).Among the sixteen tested media minimum days to callus initiation (7.33) was recorded in media containing 0.5 mgL^{-1} 2, 4-D + 0.5mgL^{-1}NAA followed by (9.00) in media with 1 mg L^{-1} 2, 4-D + 0.5mg L^{-1}NAA (Fig. 2). In contrast, more number of days to callus initiation (14.67) was taken by the explants grown on media supplemented with 0.5 mg L^{-1} 2, 4-D. Whereas, no callusing was recorded on MS basal media. The plant growth regulators 2, 4-D and NAA are widely used in plant tissue culture for callus induction (Akter *et al.*, 2008). In this study, the combination of 2, 4-D and NAA at lower concentrations was effective for the early initiation of calluses from stem segments of strawberry. Explant dedifferentiation responses to different PGRs can be explained by changes in responsiveness to media components (Ikram-ul-Haq, 2005), endogenous hormone levels (Kumar & Srivastava, 2015), and genotype (Gerszberg *et al*., 2015). Previous studies showed that low concentrations of 2,4-D in combination with NAA resulted in earlier callus initiation in Pumpkin (Pal *et al*., 2007), Egg plant (Satish *et al*., 2015) and Jojoba (Bala *et al*., 2015).

Callus induction percentage (%): On media supplemented with 0.5 mg L^{-1} 2, 4-D + 0.5 mg L^{-1} NAA maximum callus induction (93.33%) was observed, followed by (83.33 %) on media containing 1 mg L^{-1} 2, 4-D + 0.5 mg L^{-1}NAA. The lowest callusing percentage (53.33%) was recorded on media supplemented with 2.5 mg L^{-1} 2, 4-D + 1 mg L^{-1}NAA. Explants cultivated in MS basal media without growth regulators, on the other hand, did not form callus masses (Fig. 2). To induce explant differentiation and callus production *In vitro*, exogenous plant growth regulators are essential in culture media (Satish *et al*., 2015). According to Letham (1974)auxin plays a significant function in plant growth and morphogenesis by enhancing cell division, cell elongation and cell development. Both 2, 4-D and NAA are potent auxin and proved beneficial to induce callus induction frequency in the tissue culture system (Bakhtiar *et al.*, 2016). In this study, explants cultivated on media supplemented with lower concentrations of 2,4-D and NAA had a higher frequency of callus induction as compared to the higher concentrations of these two or 2,4-D alone. In other plant species, such as Coriander (Ali *et al.*, 2017), Caralluma (Zamir *et al*., 2016) and Common duckweed (Khvatkov *et al.*, 2015), the combination of 2,4-D and NAA combination favored callus induction.

Days to shoot initiation: The earlier shoot initiation (within 13.33 days) was observed in calluses cultured on media containing 1.0 mg L^{-1}TDZ + 0.5 mg L^{-1} Kin, followed by (16.00 days) on media supplemented with 2mg L^{-1} TDZ. Whereas, maximum days to shoot initiation (28.33days) were taken by calluses cultured on media added with 0.5 mg L^{-1} TDZ (Fig. 3). The composition of the media has a significant impact on the effectiveness of regeneration. Kinetin is a cytokinin that has been employed in the *In vitro* regeneration and proliferation of shoots (Anisuzzaman *et al*., 2010; Narendran *et al*., 2013). However, TDZ has recently become popular for promoting shoot regeneration and proliferation in a variety of plant species and can replace the use of other cytokinins such as BA, BAP and Kintien (Faisal *et al*., 2014). In the current study, a lower concentration of TDZ resulted the highest regeneration from strawberry callus masses. Previous studies showed that TDZ is a potent plant growth regulator and is beneficial for shoot initiation in tissue culture system (Verma & Bansal, 2014). Similarly, positive effects of TDZ on shoot regeneration have been documented in other plant species including Rapeseed (Biesaga-Kościelniak *et al.*, 2010), Travancore Persian violet (Kannan *et al*., 2007) and Corn mint (Faisal *et al.*, 2014).

Shooting frequency (%): Maximum shooting frequency (80%) was observed in media supplemented with 1.5 mg L^{-1} TDZ, followed by (71%) on media added with 2 mg L^{-1}TDZ (Fig. 3). Whereas, the lowest shooting frequency (37.33%) was observed on media containing 1.0 mgL^{-1}TDZ + 2.0 mgL^{-1} Kinetin. Plant growth regulators are essential for the plant morphogenic response. Combinations of TDZ and kinetin were found to be ineffective for multiple shoot development in this study when compared to TDZ alone. TDZ triggers a metabolic cascade that leads to changes in primary signalling pathways. It serves as a secondary messenger and transfers endogenous auxin, For example, it acts as a secondary messenger, transferring endogenous auxin and promoting morphogenesis (Biesaga-Kościelniak *et al.*, 2010). The effectiveness of TDZ on shoot regeneration may be explained by the capability of this plant growth regulator to stimulate the biosynthesis of endogenous adenine-type cytokinin (Huetteman & Preece, 1993) and/or ability to work as an efficient bioregulator of *In vitro* morphogenesis (Murthy *et al.*, 1998). Kinetin is a major cytokinin that is widely used in culture media for shoot regeneration (Barciszewski *et al*., 2007), and proved beneficial to promote shooting in Mustered (Guo *et al*., 2011).

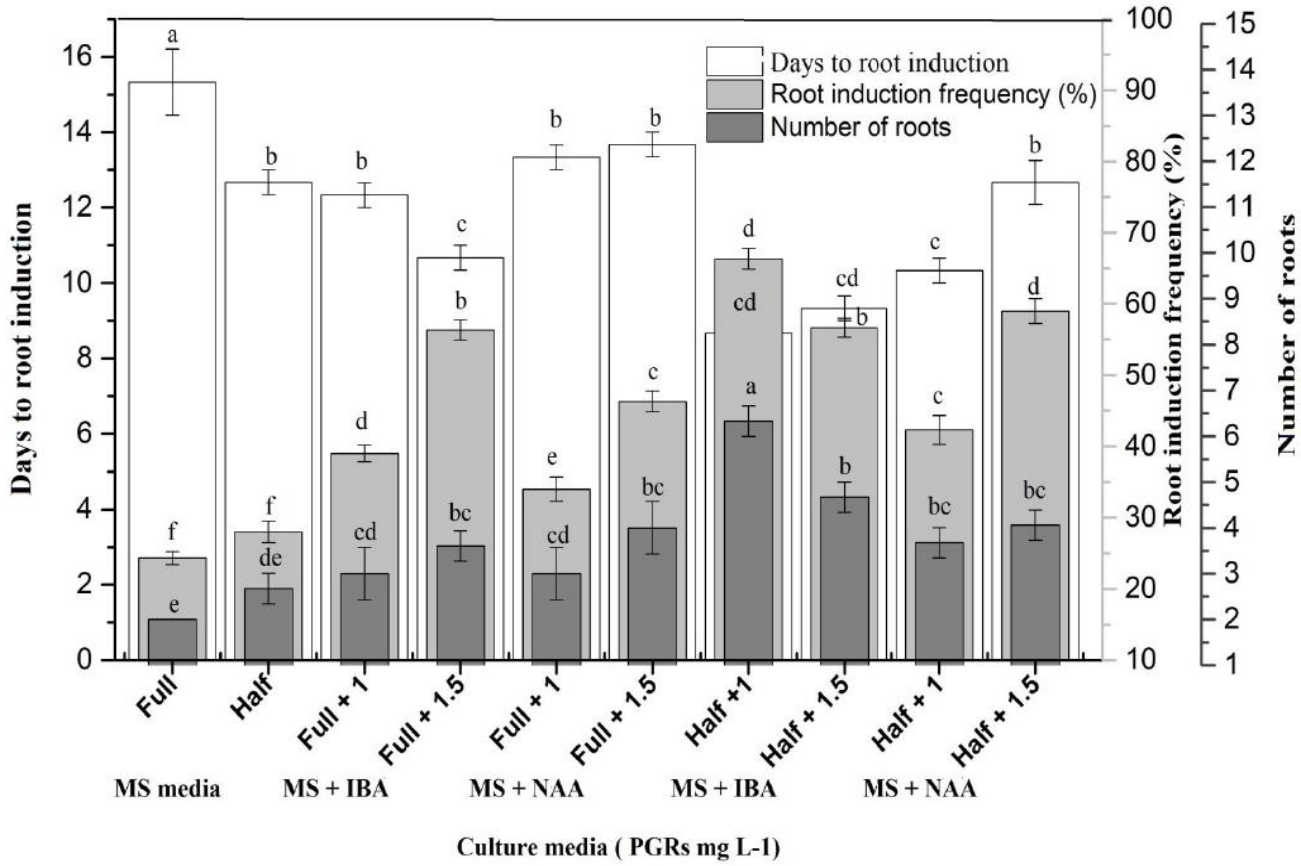

Fig. 4. Rooting from in vitro derived shoots grown on 10 different types of media, (MS) Murashige and Skoog medium, (IBA) Indole-3-butyric acid, NAA Naphthaleneacetic acid, (PGRs) Plant growth regulators. Each bar represents mean ± SE; n = 3. Different letters above the bars in respective panels indicate significant differences between the treatments at $p<0.05$ (Tukey's test).

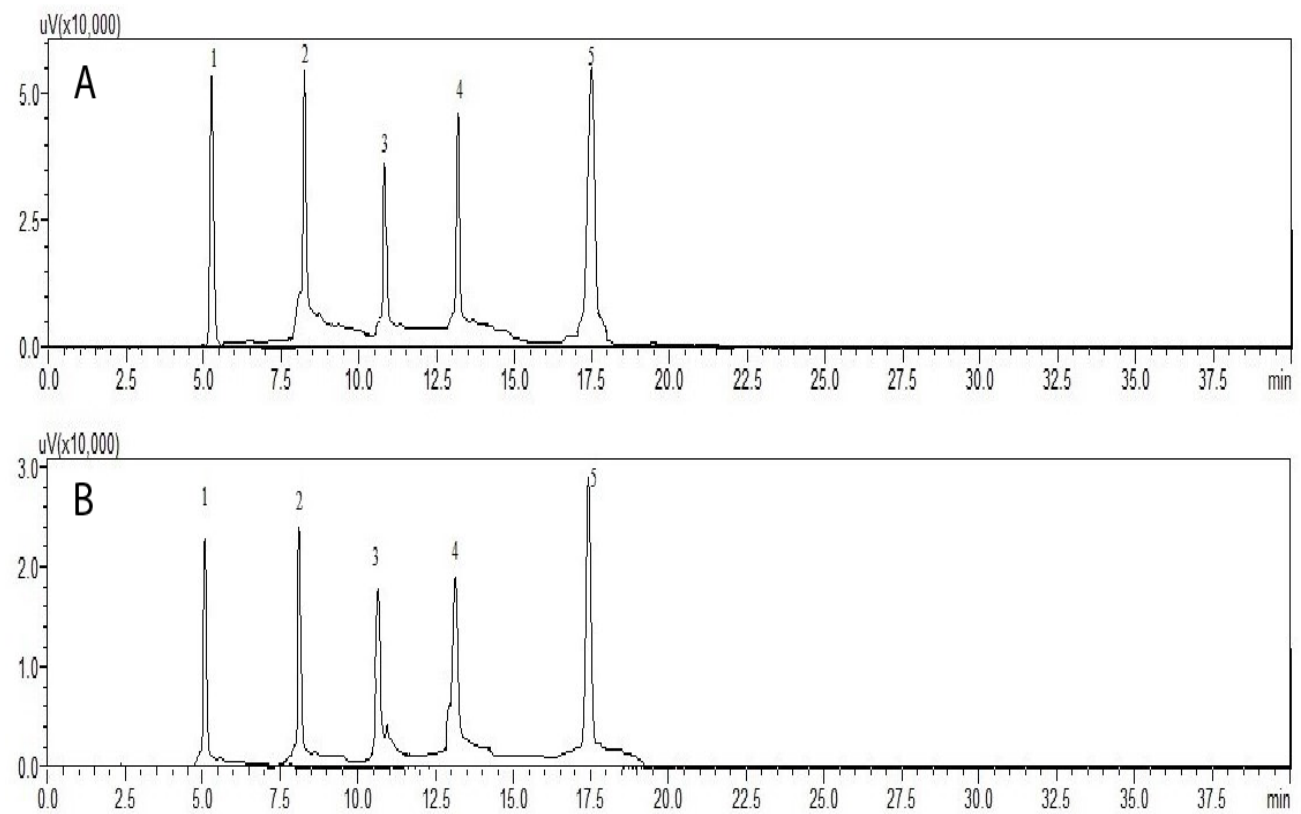

Fig. 5. HPLC chromatogram of acidified methanolic extracts from fruits of micropropagated plants (A), and from fruits of in vivo grown strawberry plants (B) recorded at 520 nm on the basis of the retention time of the authentic standards. Peaks numbers refers to Table 2.

Number of shoots developed per callus: Among the tested media more number of shoots (5.67) were recorded in calluses cultured on MS media augmented with 1.5 mg L^{-1} TDZ, followed by (5.0) in media containing 2 mg L^{-1} TDZ. Whereas, calluses cultured on MS basal media without any PGR did not produce shoots (Fig. 3).Thidaizouran is a powerful plant growth regulator that exhibits cytokinin and auxin-like properties in plant tissue culture (Folta & Dhingra, 2006). Previously, TDZ was effective to enhance the shooting of herbaceous plants including Aloe vera (Seran & Ahmad, 2018). However, kinetin was reported beneficial for shooting woody plants including apples (Dobránszki & Mendler-Drienyovszki, 2014). Similarly, Haddadi *et al*., (2010) documented a decrease in the number of shoots in strawberries with an increase in TDZ concentration from a certain level.

Days to roots induction: The early root induction (8.67 days) was observed in shoots cultured on half-strength MS media augmented with 1 mg L^{-1} IBA. Whereas, slower root induction (15.33 days) was recorded in shoots that were cultured on full strength MS basal media without the addition of PGRs (Fig. 4). In this study, rooting response was faster on half-strength MS media than on full-strength MS media. Moreover, rooting medium with IBA resulted in faster root induction as compared to NAA (Fig. 4). Nutrient deficits act as powerful stimulants for rhizogenesis (Barik *et al*., 2004). The cultured shoot cells can successfully take in water when the osmotic strength is low, but when the osmotic strength is high (i.e., full-strength MS medium), the shoot cells are unable to take in enough water, resulting in slower and restricted root growth (Satish *et al.,* 2015). In comparison to NAA, different concentrations of IBA were efficient for earlier root regeneration without the formation of basal callus. On half-strength MS medium with IBA, *In vitro* rooting was also faster in *Ficus anastasia* (Malki & Elmeer, 2009), *Pogostemon Cablin* Benth (Swamy *et al*., 2014), and *Jatropha curcas* (Rathore *et al*., 2015).

Root induction frequency (%) and number of roots plantlets^{-1}: Maximum root induction (66.33%) was noticed on half-strength MS media supplemented with 1 mg L^{-1} IBA, followed by (56.67%) on half-strength MS media added with 1.5 mg L^{-1} IBA. Moreover, more number of roots (5.8 shoot $^{-1}$) was observed on the same medium. Poor rooting response (24.33%) was observed in shoots cultured on full-strength MS media without plant growth regulators (Fig. 4). Micropropagation of many plant species is hampered by poor in-vitro rooting in the plant tissue culture system (Dewir *et al*., 2016; Oakes *et al*., 2016). Nutrient deficits media, serve as strong stimulants for rhizogenesis (Debnath, 2009).Because of the lower nitrogen content rather than the lower osmotic potential, reduced salt concentration (MS half strength) is believed to be beneficial for *In vitro* rooting (Dewir *et al.,* 2016). Indole-3-butyric acid (IBA) is a potent auxin and has been documented to accelerate rooting in plant tissue culture system (Hasan *et al*., 2010). In our study, half strength MS media with IBA was effective for maximum root initiation and more number of roots. Reduced salt concentration (1/2 MS) in the medium considerably boosted root induction in Eggplant (Satish *et al.,* 2015) and Onion (Ramakrishnan *et al.,* 2013). Previously, several plant species produced efficient roots on half-strength MS medium with IBA such as *Pogostemon cablin* Benth (Swamy *et al*., 2014), *Ficus anastasia* (Malki & Elmeer, 2009), and *Jatropha curcas* (Rathore *et al.,* 2015).

Fruit number and yield plant^{-1}: Fruit number per plant was highest (27.45) in field grown plants as compared to micropropagated plants (20.23). Similarly, field grown plants were found more yielding (222.28 g plant^{-1}) as compared to *In vitro* grown plants (192.24 g plant^{-1}) (Table 1). Plants developed via *In vitro* propagation exerted a lot of energy on growing new axillary branches and rhizomes, which could have decreased the size and weight of their fruits (Foley & Debnath, 2007). Field-grown berry plants, on the other hand, conserved energy by generating fewer rhizomes, allowing for larger fruit size and, as a result, increased berry weight (Debnath & Goyali, 2020). Previously, it was discovered that the application of cytokinins during *In vitro* propagation has an effect on vegetative development as well as fruit production in beery plant species such as cranberry (Debnath & An, 2019), lingonberry (Debnath & Arigundam, 2020) and blueberry (Marino *et al.,* 2014). In a study by Goyali *et al*., (2015) field grown lowbush blueberry plants produced better vegetative growth and yielded better than micro propagated plants. Similar trends were reported in other blue bush berry and lingonberry by Litwińczuk *et al*., (2005) and Vyas *et al*., (2013). On the other hand, higher yields of micropropagated plants were found without a reduction in fruit quality in 'Northblue' blueberry (El-Shiekh *et al.,* 1996) and lingonberry 'Sanna' cultivars (Gustavsson & Stanys, 2000). Whereas, there was no difference in the number of flower or berry weight per plant between existing field-grown and micropropagated lowbush blueberries plants (Morrison *et al*., 2000).

Biological fruit compounds of micropropagated and *In vivo* grown plants: The study revealed that fruits of micropropagated plants yielded higher anthocyanin content (126.44 mg/100 g of FW) as compared to field-grown plants (104.66 mg/100 g of FW) (Table 1). Plant tissue culture appears as a viable tool for increasing secondary metabolite levels in higher plants. Plant growth regulators used in the medium may have increased anthocyanin production by up-regulating genes involved in the flavonoid biosynthetic pathway, resulting in higher anthocyanin content in micropropagated plants (Zifkin *et al.,* 2012). Deikman & Hammer, (1995) reported higher anthocyanin content in *A.Thaliana* as a result of increased level of transcription of the genes in flavonoid biosynthesis pathway coordinately with cytokinin concentration in tissue culture system. Similar to anthocyanin, the content of total phenolic was higher (25.56 mg/100 g of FW) in fruits of *In*

vitro grown plants as compared to field-grown plants (18.44mg/100 g of FW). This result was in line with Goyali *et al*., (2015) who reported higher contents of phenolic in micropropagated blue berries as compared to conventionally grown blue berries from stem cuttings. According to Goyali *et al*., (2015) the potential of berry plants to produce polyphenols and flavonoids is influenced by propagation strategies. In their study they found higher total phenolic and flavonoid contents in the fruit of micropropagated blueberries than in plants propagated through conventional softwood cuttings. Similar to our results, higher content of phenolic in *In vitro* grown plants was reported in lingonberryVyas *et al*., (2013) and cranberry (Georgieva *et al*., 2016). In our study, it was found that ascorbic acid content was more (104.56 mg/100 g of FW) in fruits of micropropagated plants than in field-grown plants (88.68 mg/100 g of FW). According to Zushi *et al*., (2020) plants regeneration program can regulates L-Ascorbic acid (AsA) system thus can stimulate the antioxidant system. In their study higher ascorbic acid content was found in tomato fruits grown *In vitro* as compared to field-grown plants. In comparison to field-grown plants, Minutolo *et al*., (2020) found that the GDP-mannose pyrophosphorylase1 (GMP1 and l-galactono-1,4-lactone dehydrogenase (GLDH) genes involved in ASA biosynthesis were up-regulated in the fruit of micropropagated plants.

Anthocyanin pigment composition in fruits: The qualitative analysis of anthocyanin pigmentation in fruits of micropropagated and *In vivo* grown strawberry plants were conducted. The anthocyanin pigments in the fruits of micropropagated plants were identical to those in the fruits of plants grown in the field. Figure 5 shows reversed phase-HPLC-chromatograms of fruits from micropropagated and field-grown plants. Identification was attained by comparing their retention time with standard anthocyanin. According to results, the main anthocyanin in strawberry fruits are cyanidin based anthocyanins. On the chromatogram of fruit extract, five pigments were identified, with Cyanidin-3,5-O-diclocoside accounting for the majority (65.13%) of the overall anthocyanin content (Table 2). Moreover, anthocyanin concentration was higher in fruits collected from *In vitro* grown compared to field-grown fruits. The stimulatory role of micropropagation in increasing anthocyanin content may be due to the effect of plant growth regulators on phenolic compounds biosynthesis by influencing the expression or up-regulation of genes involved in secondary metabolite biosynthetic pathway (Sakakibara *et al*., 2006; Zifkin *et al*., 2012). Previously, it was discovered that the level of transcription of the gene encoding Chalcone synthase and Chalconeisomerase in the flavonoid biosynthesis pathway increased in accordance with cytokine resulting in increased concentration in anthocyanin levels in *A. thaliana* (Debnath, 2009). Similar to our findings Goyali *et al*., (2015) found that micropropagated blue berries contained more flavonoid than conventionally grown blue berries, despite the fact that both plants were grown in same greenhouse under similar environmental conditions. Other micropropagated berries such as bilberry and lingonberry showed a similar trend in anthocyanin, phenolic, and flavonoid content (Georgieva *et al*., 2016; Vyas *et al*., 2013). There are no definite reports on strawberry anthocyanin characterization from micropropagated and field grown plants that we are aware of. Similar differences in anthocyanin pigment accumulation in micropropagated plant fruits compared to traditionally produced plant/fruit have been seen in a variety of plant species, including lowbush blueberries (Goyali*et al.*, 2015) and lingonberries (Foley & Debnath, 2007).

Table 1. Quantitative and qualitative attributes of micropropagated and field-grown strawberries.

Parameters	Fruits of micropropagated plants	Fruits of field grown plants
Number of fruits plant $^{-1}$	20.23^{b}	27.45^{a}
Yield (g plant $^{-1}$)	192.24^{b}	222.28^{a}
Anthocyanin (mg/100 g of FW)	126.44^{a}	104.66^{b}
Total phenolic (mg/100 g of FW)	25.56^{a}	18.44^{b}
Ascorbic acid (mg/100 g of FW)	104.56^{a}	88.68^{b}

Different letters within each row indicate significance at ($p<0.05$) according to Tukey's test

Table 2. Anthocyanin pigments and concentration in strawberry fruits from micropropagated and field grown plants.

Peak	Anthocyanins	Retention time (min)	Calibration curve	Concentration (mg/ 100 g FW)	
				Fruits of micropropagated plants	Fruits of field grown plants
1.	Cyanidin-3-o-actylglucoside	5.437	Y= 0.738108x	14.63 ± 1.22^{d}	12.30 ± 1.48^{cd}
2.	Cyanidin-3-o-comaryglocoside	8.198	Y=0.525856x	19.47 ± 2.37^{bc}	16.17 ± 1.48^{b}
3.	Cynaidin-3-o-glucoside	10.523	Y=0.575902x	15.05 ± 1.40^{cd}	9.93 ± 2.11^{d}
4.	Cyanidin-3-o-comaryglocoside	13.198	Y=0.385184x	22.68 ± 2.37^{b}	13.30 ± 1.48^{c}
5.	Cyanidin-3,5-o-diclocoside	17.578	Y=0.262928x	55.75 ± 1.77^{a}	43.86 ± 1.57^{a}

Values are means ± standard errors of three replication; different letters in same column indicate significant differences at $p<0.05$ according to Tukey's test

Conclusion

We established an efficient *In vitro* regeneration method for Strawberry (Fragaria × ananassa) from stem segment explants via indirect organogenesis. The antioxidant metabolites study indicated that fruits from micropropagated plants could be used to extract bioactive compounds for the food, pharmaceutical, and medical industry. These findings indicate that micropropagated chandler plants can be used as mother plants to produce new chandler plants for commercial strawberry fruit production. More research into the use of strawberry micro-mother plants to develop significant nursery production in the future could give grower and consumers the opportunity to produce high quality strawberries.

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