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Original Paper

Protocol for micropropagation of strawberry (*Fragaria* × *ananassa*) cv. 'Sweet Charlie' and 'Winter Dawn'

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Abstract

The objective of this study was to develop a protocol for large-scale propagation of strawberry cv. 'Sweet Charlie' and 'Winter Dawn' using the micropropagation technique. Shoot cultures were obtained from shoot tips on Murashige and Skoog (MS) medium with 4% table sugar, 0.75% agar, 5 mg L^{-1} 6-benzyladenine and 0.01 mg L^{-1} kinetin. These shoots were multiplied and maintained on MS medium with 1 mg L^{-1} 6-benzyladenine and 0.1 mg L^{-1} kinetin. Rooting of *in vitro* raised shoots was successfully conducted by pulse treatment with 500 mg L^{-1} indole-3-butyric acid for 30 s and subsequent culturing in MS medium with 1 mg L^{-1} indole-3-butyric acid, 0.1 g L^{-1} activated charcoal and 6% table sugar. Plants were successfully acclimatized and survived in field conditions.

Key words: micropropagation, strawberry, 'Sweet Charlie', 'Winter Dawn'.
Abbreviations: AA, ascorbic acid; ADS, adenine sulphate; BA, 6-benzyladenine; IBA, indole-3-butyric acid; KN, kinetin; MS, Moorashige and Skoog; RO, reverse osmosis.

Introduction

Strawberry (*Fragaria* × *ananassa*) is a hybrid species of the genus *Fragaria*, which is grown worldwide for its fruit. The fruit is known throughout the world for its distinctive red colour, juicy texture, aroma and sweetness (Giampieri et al. 2012; Ulrich et al. 2018). Fruits are consumed either fresh or utilized in preparation of chocolates, jam, juice, ice cream, pies, and milkshakes (Afrin et al. 2016). Cultivars of strawberry have their distinct colour, flavour, size, and shape, degree of fertility, season of ripening, shelf life of fruit, disease resistance and nutrition quality (Vallarino et al. 2018). In 2017, the global production strawberries was 9.22 million tons (FAOSTAT 2017). Strawberries can be cultivated in a wide range of agro-climatic conditions (Martinelli 1992). Hence, commercial production for the fresh fruit market and processing industry has increased dramatically in last two decades (Qin et al. 2008; FAOSTAT 2017).

During the last three decades, several Florida-bred cultivars have been released and extensively planted, including 'Carmine', 'Earlibrite', 'Strawberry Festival', 'Sweet Charlie', and 'Winter Dawn'(Santos et al. 2007). Cv. 'Sweet Charlie' is popular for its delicious, sweet and nutritious fruits (Rekha et al. 2012). The fruits are firm and can withstand gentle shipping over short distances. It is resistant to fruit rot, powdery mildew and anthracnose crown (Howard 1994). In addition, it is a good choice for the home garden. Cv. 'Winter Dawn' is moderately resistant to anthracnose fruit rot, Botrytis and Colletotrichum crown rot diseases, but it produces large and easily harvestable fruit (Chandler, Dover 2009). Runners arising from axillary buds on the plant crown are traditionally utilized for the propagation of strawberries. With this method, only a limited number of plantlets can be produced, the plantlets have poor quality, and pathogens are transferred from the stock plant, which limits agricultural yield. This is particularly crucial for viral diseases, as viruses are transported via vascular bundles (Quiroz et al. 2017).

It has been reported that micropropagated plants yield more runners per plant (Mohan et al. 2005). Micropropagation of strawberry plants was developed around fifty years ago (Boxus 1974). Thereafter, many European nurseries were interested in this technique (Mohan et al. 2005), and micropropagation protocol has been utilized for commercial-scale propagation of strawberries in many countries (Boxus 1989; Martinelli 1992; Palei et al. 2015). The objective of this study was to develop a large-scale propagation protocol for strawberry cv. 'Sweet Charlie' and 'Winter Dawn' using the micropropagation technique.

Materials and methods

Development of cost-effective protocol

For development of a cost-effective protocol, some alternative strategies were attempted. To develop a cost-effective protocol, table sugar cubes from the local market, LED lights to reduce cost of electricity, low cost agar, reverse osmosis (RO) water, and laboratory grade chemicals were used (Table 1 and 2).

Disinfection of shoot tip

Aseptic cultures were initiated from 3 to 4 cm long runner tips of two-month-old healthy plants of strawberry cultivars 'Sweet Charlie' and 'Winter Dawn', which were maintained in a greenhouse during the July to September period. Shoot-tips were washed with running tap water for 10 min to remove adhering dust. Afterwards, these shoot tips were soaked in 3% Teepol™ (liquid soap solution) for 5 min and washed in running tap water for 10 min. Shoot tips were disinfected in aseptic condition with 1% bavistin (a fungicide solution consisting of carbendazin 12% + mancozeb 63%) for 10 min. Later, these shoot-tips were treated using 0.5% sodium hypochloride solution for 7 to 8 min, followed by immersion in 0.05% mercuric chloride for 1 min, and finally the shoot tips were washed three times with sterilized RO water. Shoot tips were trimmed (0.4 to 0.5 cm) at the cut end prior to inoculation onto culture initiation medium.

Culture medium

Culture medium consisted of Murashige and Skoog (1962; MS) nutrient medium containing 0.75% tissue culture grade agar (Titan Biotech, Delhi, India), 0.7 g L^{-1} ascorbic acid (AA), 10 mg L^{-1} adenine sulphate (ADS) and 4% table sugar (from a local supplier). The pH of the culture media was adjusted to 5.8, and about 50 mL medium was poured in glass bottles with semi transparent polypropylene screw-caps (300 mL volume, Hindusthan National Glass & Industries, Nashik, India) and autoclaved for 15 min at 121 °C. All chemicals used for preparation of MS medium were of laboratory grade and procured from SRL Chemicals, Mumbai, India. Shoot clumps were subcultured at regular interval of 35 days.

Culture conditions

Cultures were kept at 16:8 h light/dark photoperiod with light intensity of 2500 lux (Wipro 4-Feet 20 W LED Batten Cool Day Light, Wipro Lighting, Pune, India), and 25 ± 2 °C temperature.

Culture initiation

Culture initiation medium consisted of MS medium with additives as mentioned in the culture medium section with 5 mg L^{-1} benzyladenine (BA), 0.1 mg L^{-1} kinetin (KN), 0.7 g L^{-1} AA, 10 mg L^{-1} ADS and 4% table sugar, which produced around 4 to 5 shoots within one month.

Shoot multiplication

During culture initiation and initial subculturing (up to second subculture), only single shoot-tips or shoot clusters were inoculated per bottle to avoid culture loss due to microbial contamination. During shoot multiplication, MS medium with 1 mg L^{-1} BA and 0.01 mg L^{-1} KN was used for first two subcultures to avoid loss of cultures due to endophytic contamination (shoot clump each with five shoot buds with > 1 cm shoot length). From the third subculture, three shoot clumps (each bottle consisted of three shoot clumps with 5 to 6 shoots with > 1 cm shoot length) were maintained on 3/4 strength MS medium with 0.5 mg L^{-1} BA and 0.1 mg L^{-1} KN to avoid vitrification and stunted growth. After the ninth subculture, cultures were again initiated from micropropagated plants maintained in the greenhouse.

In vitro *rooting*

Prior to *in vitro* rooting, the cut ends of *in vitro* raised

Table 1. Cost effective micropropagation of strawberry cultivars 'Sweet Charlie' and 'Winter Dawn'

Item	Options available	Used in the present work	Cost
Light source	While fluorescent tubes / halogen bulb	LEDs used (low energy consumption)	LED lamps use 25 to 30% of the energy and last 8 to 25 times longer than halogen lamps (Energy.gov 2021)
Carbohydrate source	Sucrose	Table sugar cubes from local market	Labogen AR Grade sucrose price is 15.16 US$ per kg, while table sugar cube cost is 1 US$ per kg
Gelling agent	Agar or gelrite or phytagel	Agar-agar type II (low cost)	50% less price
Water	Distilled water	Reverse osmosis (RO) water	Distilled water systems are more expensive to operate and take longer to filter water than RO system (Applequist 2018)
Grade of chemicals	Analytical	Laboratory grade	Laboratory grade chemicals have low cost
Acclimatization matrix	Soilrite / rockwool / perlite cocopeat / moss	Only cocopeat	Cocopeat is less expensive as compared to perlite

Table 2. Strategies for the establishment of micropropagation of strawberry cultivars 'Sweet Charlie' and 'Winter Dawn'

No.	Strategy	Reason
1	Only greenhouse grown healthy plants were selected as donor plant	Problem with stock plant or disinfection procedure or subculture
2	During initial subculture one explant per culture vessel and discard infected plants at subculture stage	Useful for early detection of microbial contamination
3	From third subculture onwards: • ¾ MS medium, • benzyladenine concentration has been reduced: 0.5 mg L^{-1}, • agar concentration has been increased: 0.85%	To prevent hyperhydricity and shoot tip necrosis during subculture
4	Pulse treatment with 500 mg L^{-1} of indole-3-butyric acid and addition of activated charcoal in rooting medium	Pulse treatment improves rooting and activated charcoal provides dark environment and improves rooting in both cultivars

shoots were treated with 500 mg L^{-1} indole-3-butyric acid (IBA) solution for 30 s and then were cultured on MS medium with 1 mg L^{-1} IBA, 0.1% activated charcoal and 6% table sugar.

Acclimatization of plantlets

For primary hardening, in vitro rooted shoots were taken out from the culture medium and gently washed with tap water to eliminate any traces of adhering medium and were transferred to nursery trays (98 cavity, local market, Vadodara, India) containing sterilized cocopeat in a polyhouse. Nursery trays were covered with transparent polythene (~ 100 micron) in a low tunnel for 10 days to maintain high relative humidity (95%) and protect them from water and light stress. These plants were irrigated with 0.2% (w/v) liquid nitrogen, phosphorous and potassium fertilizer solution Sardar WSF-19-19-19TM (GSFC, Vadodara, India) at regular intervals of three days.

Secondary hardening and field transfer was performed after four weeks. The plantlets were transferred to the net-house onto plastic bags containing garden soil for acclimatization and hardening and then were transferred outdoors for the field trial. Field trials were conducted in Mahabaleshwar, India (17.93° N, 73.64° E). The field soil texture was sandy silt / sandy loam (3 to 6% ignition loss). Plants were cultivated in a well-drained medium loam soil, rich in organic matter. The pH of soil ranged from 5.7 to 6.5. It was observed that at higher pH, secondary root and runner formation was inhibited in the field. Plants were raised on 4 × 4 m or 4 × 3 m beds. The distance between two plants was 45 cm and about 60 to 75 cm between two rows. These plants were irrigated at 25- to 28-day intervals during evening hours.

Results

This study presents the developed micropropagation protocol for strawberry cv. 'Sweet Charlie' and 'Winter Dawn using shoot tip explants. On the culture initiation medium, about 4 to 5 shoot buds developed from the petiole base of the older leaves in all cultures within 3 to 4 weeks. These shoot buds grew very quickly and produced new shoot buds continuously on the same medium. Within 2 months, these cultures turned into clusters of small shoot buds.

Repeated subcultures of shoot clumps on 3/4 strength MS with 0.5 mg L^{-1} BA and 0.1 mg L^{-1} KN resulted in rapid shoot multiplication. It was observed that, during subculture, the shoot multiplication rate of the 'Winter Dawn' cultivar was about 15% higher than for 'Sweet Charlie. However, after the ninth subculture, *in vitro* cultures showed sign of vitrification and decrease in shoot length, and there was a decline of the survival rate of plants in a natural condition.

Pulse treatment with IBA and subsequent culturing in MS with 1 mg L^{-1} IBA and 0.1% activated charcoal resulted in 100% root formation within 25 days (Table 2). The plants began to produce runners during secondary hardening and were ready for field planting after 4 months.

Discussion

For the commercial-scale production of strawberry plantlets, the micropropagation technique is most popular method (Boxus 1989; Capocasa et al. 2019). Adams (1972) was the first to demonstrate the possibilities of propagation of strawberry plants using shoot tip meristems. Apical meristem culture is the only method that ensures genetic uniformity of micropropagated plants (D'Amato 1977). It is difficult to establish aseptic culture from field-grown plants due to a high contamination rate. Explants collected from plants grown in the greenhouse show more regeneration potential, high viability and minimal contamination, than for other methods. (Niedz, Bausher 2002; Fraga et al. 2004; Hand et al. 2016). Therefore, shoot tips from greenhouse grown healthy plants are utilized as for micropropagation of strawberry.

Repeated subculturing of shoot clumps helped to achieve continuous shoot production. However, after repeated subculturing, occurrence of vitrification can be due to either genetic or epigenetic changes resulting from repeated fluxes in cytokinin, nutrient status or sucrose, or from elimination of seasonal environmental fluctuations

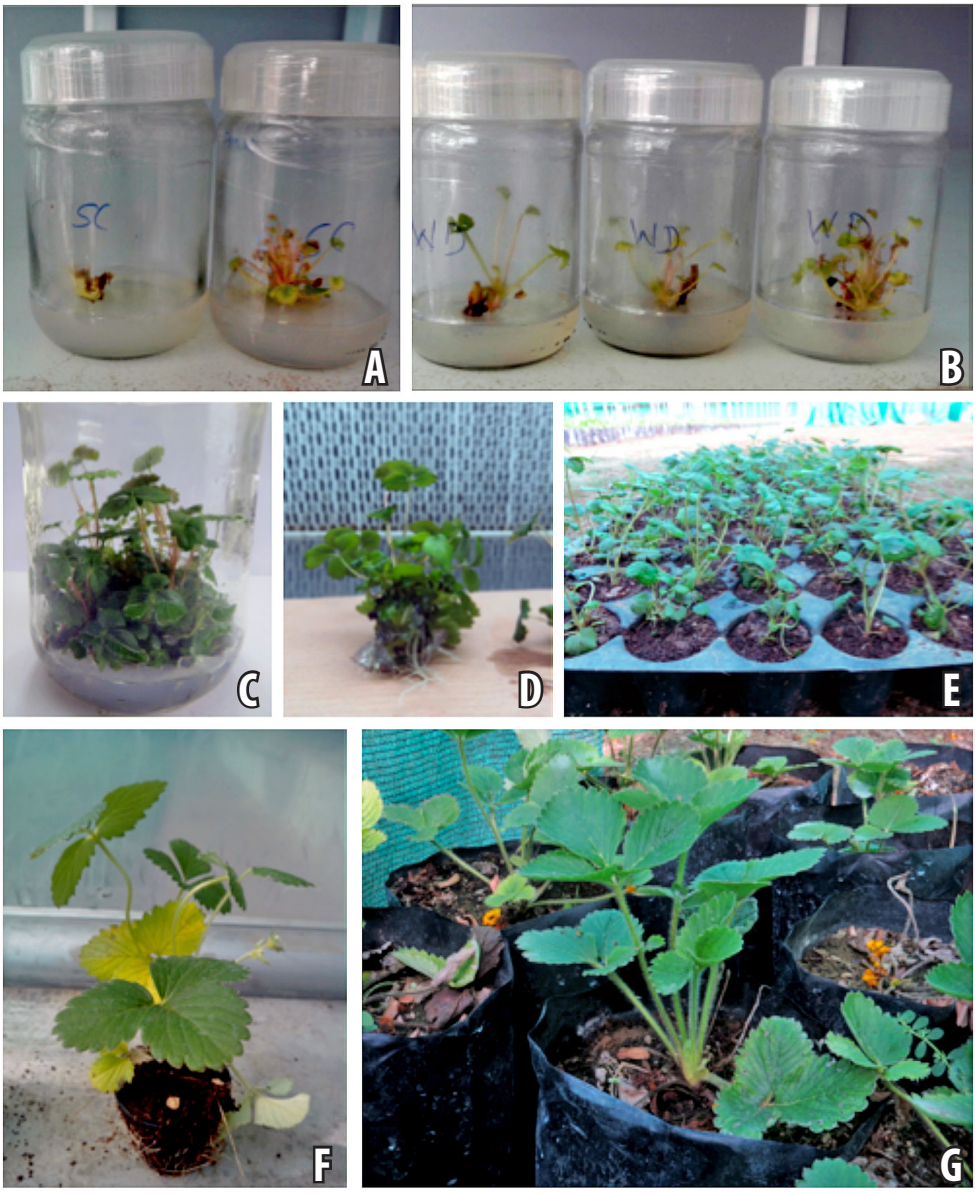

Fig. 1. Micropropagation of strawberry cultivars 'Sweet Charlie' and 'Winter Dawn'. A, cv. 'Sweet Charlie' culture initiation (MS medium + 5 mg L^{-1} benzyladenine + 0.1 mg L^{-1} kinetin + 10 mg L^{-1} adenine sulphate + 0.7 g L^{-1} ascorbic acid + 4% table sugar + 0.75% tissue culture grade agar). B, cv. 'Winter Dawn' culture initiation (MS medium + 5 mg L^{-1} benzyladenine + 0.1 mg L^{-1} kinetin + 10 mg L^{-1} adenine sulphate + 0.7 g L^{-1} ascorbic acid + 4% table sugar + 0.75% tissue culture grade agar). C, shoot multiplication (cv. 'Sweet Charlie': 3/4 MS + 0.5 mg L^{-1} benzyladenine + 0.1 mg L^{-1} kinetin + 10 mg L^{-1} adenine sulphate + 0.7 g L^{-1} ascorbic acid + 4% table sugar + 0.75% tissue culture grade agar). D, *in vitro* rooting (cv. 'Sweet Charlie': *in vitro* raised shoots pulse treated with 500 mg L^{-1} indole-3-butyric acid for 30 s, cultured on MS with 1 mg L^{-1} indole-3-butyric acid, 0.1% activated charcoal and 6% table sugar. E, acclimatization in nursery tray with cocopit (cv. 'Sweet Charlie'). F, acclimatized plant after 1 month (cv. 'Sweet Charlie') G, acclimatized plants in nursary polybag with soil (cv. 'Sweet Charlie').

(Norton, Norton 1986).

Exogenous application of auxin stimulates rooting (Label et al. 1989), and therefore we tried pulse treatment of IBA. Efficacy of pulse treatment for *in vitro* rooting was reported for two strawberry cvs., 'Alpha' and 'Festivalnaya' (Ambros et al. 2018). IBA is extensively utilized for the *in vitro* rooting of various strawberry cultivars, including cv. 'Ofra' (Anuradha et al. 2016), 'Festival' (Sharma, Kumar 2012; Khalil et al. 2019), 'Oso Grande' (Lal et al. 2003; Rattanpal et al. 2011), 'Chandler' (Lal et al. 2003), 'CamaRosa' (Lal et al. 2003; Haddadi et al. 2010) and 'Sweet Charlie' (Sharma, Kumar 2012; Harugade et al. 2014; Khalil et al. 2019). Efficacy of IBA and activated charcoal for *in vitro* root formation was reported for cvs. 'Festival' and 'Sweet Charlie' of strawberry (Khalil et al. 2019). Activated charcoal provides a darkened condition, which promotes rooting (Table 2). However, elucidation of the exact mechanism of action of activated charcoal requires more

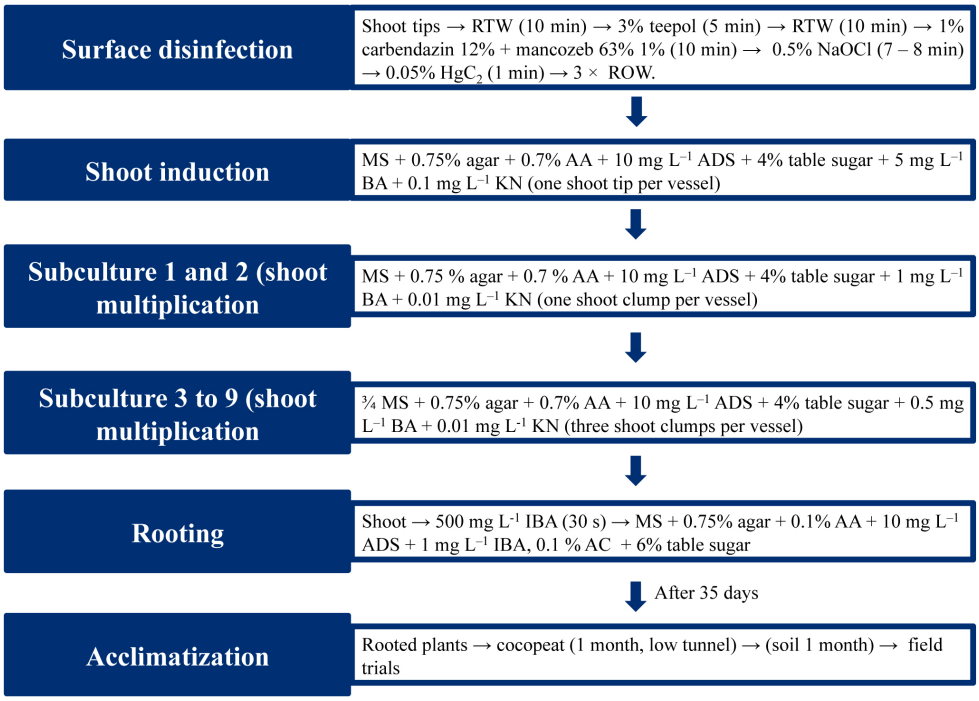

Fig. 2. Schemetic summary of micropropagation of strawberry cultivars. AA, ascorbic acid; AC, activatec charcoal; ADS, adenine sulphate; BA, 6-benzylaminopurine; $HgCl_2$, mercuric chloride; IBA, indole-3-butyric acid; KN, kinetin; NaOCl, sodium hypocloride; ROW, reverse osmosis water; RTW, running tap water.

studies (Thomas 2008).

In the present study, 95% survival of plants were observed during primary hardening on cocopeat. In contrast, combinations of perlite, vermiculite, and cocopeat (2:1:2, v/v/v) resulted in 90% plant survival and after irrigation using Hoagland's solution for cv. 'Camarosa' (Haddadi et al. 2010).

Conclusions

A method was developed for rapid shoot multiplication of two commercially important straberry cultivars, 'Sweet Charlie' and 'Winter Dawn', using shoot tip explants. Using this technique, a large number of high quality planting material can be obtained in a very short period. With the help of this protocol, more than ten thousand plants can be produced from single shoot tip within one year. The suggested protocol is summarized in Fig. 2. During the acclimatization step, 95% survival was obtained, which could reduce the costs.

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References

Adams A.N. 1972. An improved medium for strawberry meristem culture. *J. Hortic. Sci.* 47: 263–264.

Afrin S., Gasparrini, M., Forbes-Hernandez T.Y., Reboredo-Rodriguez P., Mezzetti B., Varela-López A., Giampieri F., Battino M. 2016. Promising health benefits of the strawberry: A focus on clinical studies. *J. Agric. Food Chem.* 64: 4435–4449.

Ambros E. V., Toluzakova S.Y., Shrainer L.S., Trofimova E.G., Novikova T.I. 2018. An innovative approach to *ex vitro* rooting and acclimatization of *Fragaria* × *ananassa* Duch. microshoots using a biogenic silica- and green-tea-catechin-based mechanocomposite. *In Vitro Cell. Dev. Biol. Plant* 54: 436–443.

Anuradha B., Sehrawat S.K., Poonia A.K., Kajla S., Bhat S. 2016. Production of strawberry plant by *in vitro* propagation. *Res. Crops* 17: 545–549.

Applequist B. 2018. What's the difference between RO and DI water purification? https://www.labconco.com/articles/whats-the-difference-between-ro-and-di-water-pur /accessed 22 February 2021/

Boxus P. 1989. Review on strawberry mass propagation. *Acta Hortic.* 265: 309–320.

Boxus P. 1974. The production of strawberry plants by *in vitro* micro-propagation. *J. Hortic. Sci.* 49: 209–210.

Capocasa F., Balducci F., Marcellini M., Bernardini D., Navacchi O., Mezzetti B. 2019. Comparing nursery behavior, field plant yield and fruit quality of *in vitro* and *in vivo* propagated strawberry mother plants. *Plant Cell Tissue Organ Cult.* 136: 65–74.

Chandler C.K., Dover F.L. 2009. "Winter Dawn" strawberry plant. US Pat. "US 2009/0013438 P1."

D'Amato F. 1977. Cytogenetics of differentiation in tissue and cell cultures. In: Reinert J., Bajaj Y.P.S. (eds.) *Plant Cell, Tissue, and Organ Culture*. Springer-Verlag, New York, pp. 343–357.

Energy.Gov 2021. Lighting choices to save you money. https://

www.energy.gov/energysaver/save-electricity-and-fuel/lighting-choices-save-you-money#:~:text=LEDs%20use%2025%25%E2%80%9330%25,times%20longer%20than%20halogen%20incandescents /accessed 22 February 2021/

FAOSTAT. 2017. World production of strawberries. http://www.fao.org/faostat/en/?#data/QC/visualize /accessed 22 February 2021/

Fraga M., Alonso M., Borja M. 2004. Shoot regeneration rates of perennial phlox are dependant on cultivar and explant type. *HortScience* 39: 1373–1377.

Giampieri F., Tulipani S., Alvarez-Suarez J.M., Quiles J.L., Mezzetti B., Battino M. 2012. The strawberry: Composition, nutritional quality, and impact on human health. *Nutrition* 28: 9–19.

Haddadi F., Aziz M.A., Saleh G., Rashid A.A., Kamaladini H. 2010. Micropropagation of strawberry cv. camarosa: Prolific shoot regeneration from *in vitro* shoot tips using thidiazuron with n6-benzylamino-purine. *HortScience* 45: 453–456.

Hand C.R., Wada N., Stockwell V., Reed B.M. 2016. Node position influences viability and contamination in hazelnut shoot explants. *In Vitro Cell. Dev. Biol. Plant* 52: 580–589.

Harugade S., Tabe R.H., Chaphalkar S. 2014. Micropropagation of strawberry (*Fragaria* × *ananassa* Duch.). *Int. J. Curr. Microbiol. Appl. Sci.* 3: 344–347.

Howard C.M. 1994. Strawberry plant called "Sweet Charlie." US Pat. "US00PP08729P."

Khalil H.-A., Khalil M., Arisha H., El-Denary M. 2019. Effect of cultivars, auxins and activated charcoal on *in vitro* roots formation of strawberry plantlets. *Zagazig J. Agric. Res.* 46: 609–620.

Label P., Sotta B., Miginiac E. 1989. Endogenous levels of abscisic acid and indole-3-acetic acid during *in vitro* rooting of wild cherry explants produced by micropropagation. *Plant Growth Regul.* 8: 325–333.

Lal M., Sharma S., Hegde M. 2003. Micropropagation of strawberry (*Fragaria* × *ananassa* Duch.). *Indian J. Agric. Res.* 37: 231–234.

Martinelli A. 1992. Micropropagation of strawberry (*Fragaria* spp.). In: Bajaj Y.P.S. (ed.) *Biotechnology in Agriculture and Forestry.* Vol. 18. Springer-Verlag, Berlin–Heidelberg, pp. 354–370.

Mohan R., Chui E.A., Biasi L.A., Soccol C.R. 2005. Alternative *in vitro* propagation: Use of sugarcane bagasse as a low cost support material during rooting stage of strawberry cv. Dover. *Brazilian Arch. Biol. Technol.* 48: 37–42.

Murashige T., Skoog F. 1962. A revised medium for rapid growth and bioassays with tobacco tissue cultures. *Physiol. Plant.* 15: 473–497.

Niedz R.P., Bausher M.G. 2002. Control of *in vitro* contamination of explants from greenhouse- and field-grown trees. *In Vitro Cell. Dev. Biol. Plant* 38: 468–471.

Norton M.E., Norton C.R. 1986. Change in shoot proliferation with repeated *in vitro* subculture of shoots of woody species of Rosaceae. *Plant Cell Tissue Organ Cult.* 5: 187–197.

Palei S., Das A., Rout G. 2015. *In vitro* studies of strawberry – an important fruit crop: a review. *J. Plant Sci. Res.* 31: 115–131.

Qin Y., Teixeira da Silva J.A., Zhang L., Zhang S. 2008. Transgenic strawberry: State of the art for improved traits. *Biotechnol. Adv.* 26: 219–232.

Quiroz K.A., Berríos M., Carrasco B., Retamales J.B., Caligari P.D.S., García-Gonzáles R. 2017. Meristem culture and subsequent micropropagation of Chilean strawberry (*Fragaria chiloensis* (L.) Duch.). *Biol. Res.* 50: 1–11.

Rattanpal H.S., Gill M.I.S., Sangwan A.K. 2011. Micropropagation of strawberry through meristem culture. *Acta Hortic.* 890: 149–154.

Rekha R., Mandave P., Meti N. 2012. Micropropagation of strawberry cultivar Sweet Charlie through axillary shoot proliferation. *J. Appl. Hortic.* 14: 71–73.

Santos B.M., Chandler C.K., Olson S.M., Olczyk T.W. 2007. Performance of strawberry cultivars in Florida. *Proc. Florida State Hortic. Soc.* 120: 155–156.

Sharma N.K., Kumar M. 2012. EST SSR markers analysis of In vitro regenerated plantlets of festival and sweet charlie cultivars of strawberry. *Int. J. Agric. Env. Biotech.* 5: 315–322.

Thomas T.D. 2008. The role of activated charcoal in plant tissue culture. *Biotechnol. Adv.* 26: 618–631.

Ulrich D., Kecke S., Olbricht K. 2018. What do we know about the chemistry of strawberry aroma? *J. Agric. Food Chem.* 66: 3291–3301.

Vallarino J.G., de Abreu e Lima F., Soria C., Tong H., Pott D.M., Willmitzer L., Fernie A.R., Nikoloski Z., Osorio S. 2018. Genetic diversity of strawberry germplasm using metabolomic biomarkers. *Sci. Rep.* 8: 14386.

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