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COCONUT WATER PROMOTES *IN VITRO* SHOOT MULTIPLICATION RATE FROM STRAWBERRY (*Fragaria × ananassa*) NODAL SEGMENTS

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ABSTRACT

In vitro **micropropagation of strawberry cv. Chandler was carried out from nodal segments. Among different disinfectants used, treatment of segments with mercuric chloride (0.1 % w/v) for 5 seconds showed 90 % establishment of segments. Among different media tested, solid media containing Benzyl Amino Purine (0.2 mg/L), Indole Acetic Acid (0.2 mg/L), Kinetin (0.3 mg/L), Table sugar / Sucrose (30 g/L), Coconut water (150 ml/L), Agar (8 g/L) were found to be most effective for shoot multiplication, and root formation occurred within two weeks. The liquid medium containing BAP (0.2 mg/L), IAA (0.2 mg/L), Kin (0.3 mg/L) and Sucrose (30 g/L) was found to be at par with the above medium with respect to shoot multiplication, and root formation occurred in 10 days on this medium. Plants grown in leguminous soil showed more than 90 % survival in the screen house.**

Keywords: Coconut water, Hardening, *In vitro* propagation, Root formation, Shoot regeneration, Strawberry

The garden strawberry, *Fragaria* × *ananassa* (Rosaceae) is a widely-grown hybrid species belonging to genus *Fragaria* and having a genome size of 805.5 Mb (Edger *et al*., 2019). Strawberry, nicknamed as “Queen of Fruits”, is an important crop as it bears attractive red color, fragrant and tasty fruits (Lal and Sharma, 2003) that are of commercial value in food industry. Strawberry fruits are rich in iron, copper, phosphorus, magnesium, potassium, dietary fiber, Vitamin C, antioxidants, folate, flavonoids and protect against tumor formation (Giampieri *et al*., 2012). The crop is grown world-wide mainly in temperate, Mediterranean and sub-tropical zones (Hancock *et al*., 1991). In India, strawberry is commercially cultivated in Maharashtra, Himachal Pradesh, Uttarakhand, Punjab, Haryana, Western Uttar Pradesh and Madhya Pradesh (http://nhb.gov.in/report_files/strawberry).

Strawberry can be propagated through different methods e.g. from seeds, runners or by dividing and replanting of lateral crowns. Strawberry propagation through seeds is tedious as seed size is small and difficult to handle, more over plants produced from seeds are not true-to-type. Propagation of strawberry through crown division also has certain limitations e.g. it is labor intensive, technical and plants so obtained have decreased yield. So, the crop is usually propagated using runners as it is the easiest and quickest way to develop a large number of disease-free and genetically identical plants true-to-type to the mother / stock plant (Debnath, 2013). Thus, availability of healthy stock plants and large number of runners are a pre-requisite for successful propagation of strawberry (Haddadi *et al*., 2010). However, due to accumulation of pathogens generation over generation, the use of runners is not always feasible. In this regard, *in vitro* propagation offers a reliable approach for large scale production of clean planting material of strawberry on a commercial scale.

In comparison to plants raised conventionally from runners, micropropagated strawberry plants have better characters, such as increased crown size, number of runners, early flowering and higher yield of berries (Karhu and Hakala, 2007). A large number of explants have been used for micropropagation of strawberry e.g. efficient *in vitro* shoot regeneration has been documented from leaf and runner tissues of strawberry plants by Liu and Sanford (1988). Likewise, complete strawberry plantlets have been regenerated *in vitro* from anthers (Laneri and Damiano, 1980), immature embryos (Wang *et al*., 1984) and meristematic callus (Jones *et al*., 1988).

In the present study, a simple micropropagation protocol has been outlined to produce strawberry plantlets from nodal segments of runners, excised from healthy plants of strawberry *cv.* Chandler maintained under controlled conditions.

MATERIALS AND METHODS

Plant material

Strawberry (*Fragaria* × *ananassa*) *cv.* Chandler plants (Fig 1 A) procured from a private nursery were used for collecting the nodal segments from runner portion (Fig 1 B). The nodal segments were used

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as starting material for *in vitro* micropropagation of strawberry.

Sterilization of explants

The nodal segments were washed under running tap water for 30 seconds, reduced to 2.5 cm size with the help of a scalpel and immersed in 2 % teepol solution for 5 minutes with continuous shaking to remove dust particles. This was followed by treating the segments in 2 % (w/v) carbendazim solution for 20 minutes and rinsing with sterile distilled water. Thereafter, the nodal segments were disinfected under Laminar air flow cabinet by either using 70 % (v/v) ethanol for 30 seconds or 100 % ethanol (v/v) for 30 seconds or 0.1 % (w/v) mercuric chloride for 5 seconds or 30 seconds (Table 1). Finally, the segments were washed thrice with sterile distilled water.

Inoculation and incubation of explants

Sterilized nodal segments were cultured in jam jars (250 ml capacity) containing MS (Murashige and Skoog, 1962) media augmented with Benzyl Amino Purine (BAP, 0.2 mg/L), Kinetin (Kin, 0.3 mg/L) and Agar (8 g/L). The pH of the media was adjusted to 5.8 with 0.1N sodium hydroxide before autoclaving at 1.06 kg/cm^3 and 121 °C for 20 minutes. All the chemicals were procured from Hi Media, Mumbai. The cultured segments were incubated at 25 ± 2 °C under 16 hour photoperiod of fluorescent light for further growth.

In vitro regeneration

The *in vitro* regenerated shoots were sub-cultured on various MS media having variation based on the concentration of growth regulators, addition of Coconut water, Sucrose or Table sugar (Table 2). Data were recorded on shoot multiplication, days to root formation and length of roots. Fully grown strawberry plantlets were taken out from the culture vessels for hardening.

Hardening of plantlets

For carrying out of hardening plantlets, these were first washed under running tap water to remove the media adhering to roots, and then transferred on water-soaked cotton in a plastic tray. The plantlets in the tray were covered with a polythene sheet and kept in incubation room at 25 ± 2 °C for 4 days. Thereafter, plantlets were transplanted in two types of potting mixtures, namely field soil and soil collected from chickpea field (leguminous soil), and placed in screen house for further growth.

RESULTS AND DISCUSSION

Nodal segments (Fig. 1 C) excised from runner portion of strawberry plants were treated with two different disinfectants for varying durations. Out of the two disinfectants tested, treatment of segments with mercuric chloride (0.1 % w/v) for 5 seconds gave best result with explant establishment rate of 90 % (Table 1). Diengngan *et al*. (2014) reported minimal browning and minimal contamination of strawberry explants treated with 0.1 % $HgCl_2$ for 3.10 minutes. In our case, the treatment of nodal explants with mercuric chloride (0.1 % w/v) for 30 seconds led to browning of segments, and treatment with 70 % or 100 % (v/v) ethanol resulted in bacterial contamination (Table 1). The shoot formation was observed after 3 days of culturing (Fig. 1 D) nodal segments on the establishment medium {MS medium augmented with BAP (0.2 mg/L), Kin (0.3 mg/L) and Agar (8 g/L)}, along with proliferation of shoots after 10 days of culturing segments (Fig. 1 E, F).

The shoots so obtained were sub-cultured on nine different media (Table 2) to find out the best medium for shoot multiplication. The result revealed that rate of shoot multiplication after 30 days of sub-culturing shoots was different on different media ranging from 2.20 to 3.80 (Table 3). Among various media, the best response towards rate of shoot multiplication was observed on T5 medium {MS supplemented with BAP (0.2 mg/L), Indole Acetic Acid (IAA, 0.2 mg/L), Kin (0.3 mg/L), Sucrose (30 g/L) and Coconut water (150 ml/L)} [Fig. 1 G]. Statistical analysis of the data (Table 3) revealed that rate of shoot multiplication on above-mentioned medium (3.80 ± 0.22) was significantly at par with frequency of shoot multiplication on T2 (3.61 ± 0.40) and T4 (3.65 ± 0.23) media, and was significantly better than the remaining media (T1, T3, T6, T7, T8). A decrease in the concentration of BAP (from 0.2 mg/L to 0.1 mg/L) and Kin (from 0.3 mg/L to 0.1 mg/L) in T6, T7 and T8 media led to reduced rate of shoot multiplication on these media, thus underlining the importance of cytokinins in shoot proliferation. It has been reported that cytokinins play a key part in controlling shoot branching (Haddadi *et al*., 2010). Sakila *et al*. (2007) performed a similar experiment resulting in highest number of strawberry shoots per explant on MS media supplemented with 1.5 mg/L Benzyl Adenine (BA) + 0.5 mg/L Kin after 5 weeks of culturing explants. Jhajhra *et al*. (2018) examined the effect of different combination of growth regulators on *in vitro* propagation of strawberry and observed maximum number of shoots (3.66 per explant) in media supplemented with 1 mg/L BA, 0.1 mg/L Naphthalene Acetic Acid , 1 mg/L Adenine sulphate, Sucrose (30 g/L) and Coconut water (150 ml/L). To our observation, plantlets regenerated on medium supplemented with Coconut water and Sucrose had stouter stem and wider leaves as compared to plantlets regenerated on media lacking Coconut water. This may be due to the fact that Coconut water contains several growth promoting compounds *viz*., cytokinins, micronutrients,

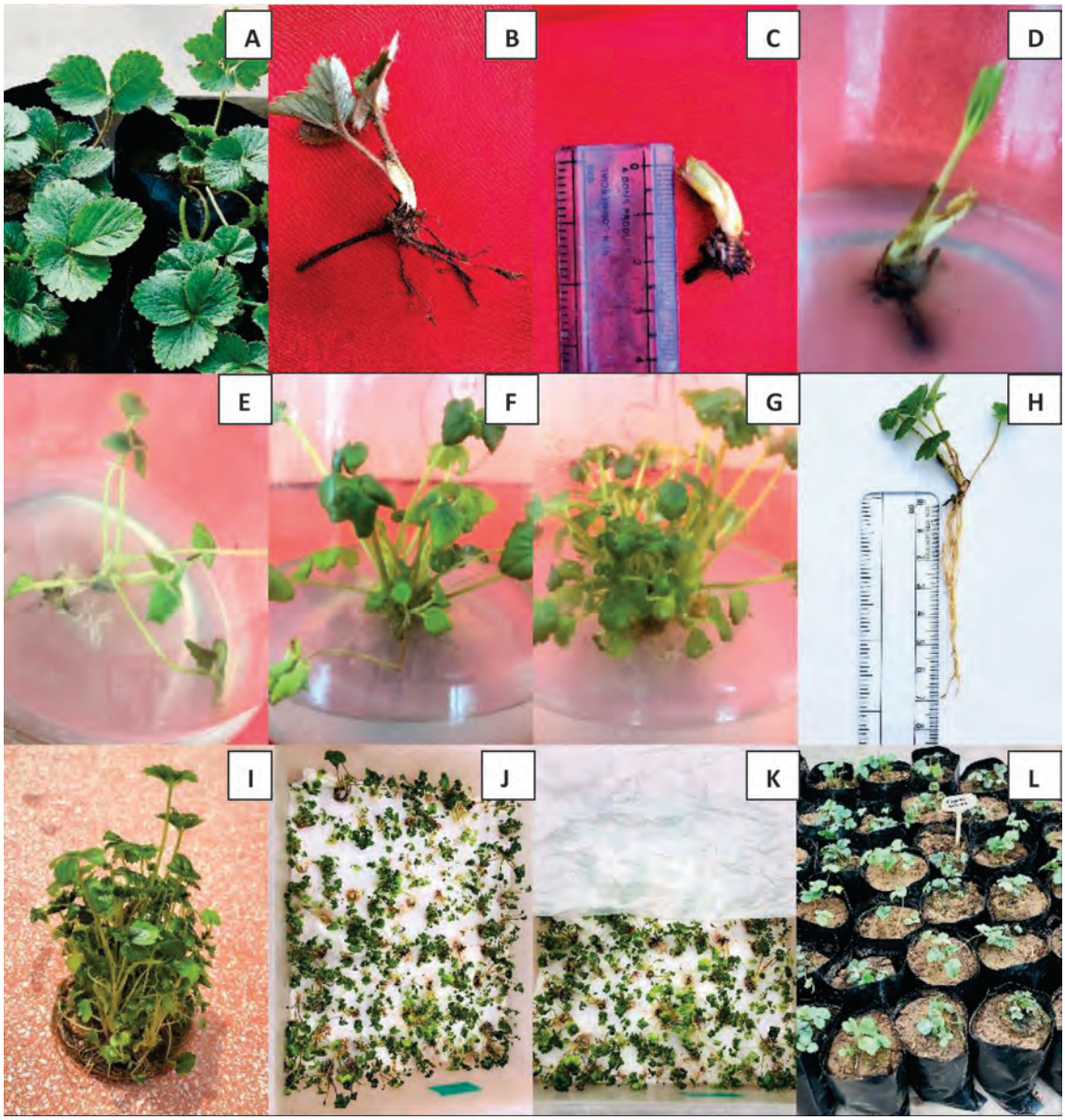

Fig. 1. Micropropagation of strawberry *cv.* Chandler from nodal segments. A: Strawberry plants procured from nursery B: Up-rooted strawberry plant showing runner and nodal portion C: Excised nodal segment measuring 2.5 cm D: Shoot regeneration after 3 days of culturing nodal segment E: Shoot proliferation from cultured nodal segment F: Shoot proliferation after 10 days of culturing nodal segment G: Shoot proliferation after 30 days of culturing H: Root formation after 30 days I: Clump of plantlets formed under *in vitro* conditions J, K: Hardening of plantlets on water-soaked cotton and their coverage with a polythene sheet L: Transplanting of plantlets in leguminous soil

Table 1. Effect of treatment of different disinfectants on establishment of nodal segments *in vitro*, excised from runner portion of strawberry *cv.* Chandler plants

Treatment	Total no. of segments cultured	No. of contaminated segments	No. of dead (brown) segments	No. of effective segments	Establishment rate (%)
70 % (v/v) Ethanol (30 sec)	20	20	-	-	0
100 % (v/v) Ethanol (30 sec)	16	14	-	2	12.5
0.1 % (w/v) $HgCl_2$ (30 sec)	24	-	24	-	0
0.1 % (w/v) $HgCl_2$ (5 sec)	20	2	-	18	90

Table 2. Composition of different MS media used for sub culturing of strawberry *cv.* Chandler shoots

Treatment	Medium composition
T1 (Control)	MS + BAP (0.2 mg/L) + IAA (0.2 mg/L) + Kin (0.3 mg/L) + Sucrose (30 g/L) + Agar (8 g/L) {SOLID}
T2	MS + BAP (0.2 mg/L) + IAA (0.2 mg/L) + Kin (0.3 mg/L) + Sucrose (30 g/L) {LIQUID}
T3	MS + BAP (0.2 mg/L) + IAA (0.2 mg/L) + Kin (0.3 mg/L) + Table sugar (30 g/L) + Agar (8 g/L) {SOLID}
T4	MS + BAP (0.2 mg/L) + IAA (0.2 mg/L) + Kin (0.3 mg/L) + Table sugar (30 g/L) + Coconut water (150 ml/L) + Agar (8 g/L) {SOLID}
T5	MS + BAP (0.2 mg/L) + IAA (0.2 mg/L) + Kin (0.3 mg/L) + Sucrose (30 g/L) + Coconut water (150 ml/L) + Agar (8 g/L) {SOLID}
T6	MS + BAP (0.2 mg/L) + IAA (0.2 mg/L) + Kin (0.3 mg/L) + Coconut water (150 ml/L) + Agar (8 g/L) {SOLID}
T7	MS + BAP (0.1 mg/L) + IAA (0.2 mg/L) + Kin (0.1 mg/L) + Table sugar (30 g/L) + Coconut water 150 ml/L) + Agar (8 g/L) {SOLID}
T8	MS + BAP (0.1 mg/L) + IAA (0.2 mg/L) + Kin (0.1 mg/L) + Sucrose (30 g/L) + Coconut water (150 ml/L) + Agar (8 g/L) {SOLID}
T9	MS + BAP (0.1 mg/L) + IAA (0.2 mg/L) + Kin (0.1 mg/L) + Table sugar (30 g/L) + Agar (8 g/L) {SOLID}

sugars, sugar alcohols, lipids, amino acids, nitrogenous compounds, organic acids and enzymes (Yong *et al*., 2009). The cytokinins in Coconut water play a major role in cell division and organ formation (Razdan, 2003).

In the present investigation, root formation in the shoots was observed to occur on same media in which shoots were sub cultured. On the liquid medium, T2 containing BAP (0.2 mg/L), IAA (0.2 mg/L), Kin (0.3 mg/L) and Sucrose (30 g/L), root initiation was seen in minimum time period, i.e. after 10 days of sub culturing shoots. On the same medium, maximum length (6.76 cm) of roots (Fig.1 H) was also observed. The quick root induction and elongation might have occurred due to better diffusion rate leading to easy availability of nutrients and water from liquid medium (Ascough and Fennell, 2004). Sakila *et al*. (2007) reported maximum rooting frequency in strawberry shoots cultured on medium containing 1.0 mg/L Indole Butyric Acid. Root formation in strawberry shoots cultured on T4 and T5 media occurred within a period of two weeks.

Thus, this study demonstrated three different types of media (T2, T4 and T5) to be effective with regard to both shoot multiplication and root formation. However, it has been reported that the use of liquid media in tissue culture may encourage vitrification of shoot cultures (Ziv, 1986; Ziv *et al*., 1987). Further, Hdider and Desjardins (1993) observed vitrification of micropropagated strawberry shoot cultures proliferated on liquid medium after a week of culturing, and advocated the addition of antivitrifying agents for preventing this problem. Out of the two media T4 and T5 used in our study, it will be more economical to work with T4 medium than T5 medium as in T4 medium table sugar is used, which is much cheaper than sucrose.

A clump of strawberry plantlets were obtained (Fig. 1 I) that were separated for carrying out hardening (Fig.1 J, K). The plantlets were then grown in potting mixture/soil (Fig. 1 L). The potting mixture collected from chickpea field (leguminous soil) showed more than 90 % plant survival, whereas in field soil, plants showed

Table 3. Effect of different culturing media on shoot multiplication rate and rooting in strawberry *cv.* Chandler

Culturing medium	Rate of shoot multiplication after 30 days of transferring initial number of shoots*	Days to root initiation	Average length (cm) of roots after 30 days
T1	3.15 ± 0.22	14	6.06
T2	3.61 ± 0.40	10	6.76
T3	2.82 ± 0.50	14	5.54
T4	3.65 ± 0.23	13	5.97
T5	3.80 ± 0.22	12	6.12
T6	3.05 ± 0.10	14	5.02
T7	2.42 ± 0.34	12	5.53
T8	2.54 ± 0.03	12	5.81
T9	2.20 ± 0.25	15	5.38

*Data on rate of shoot multiplication is presented as mean ± standard deviation of two replicates.

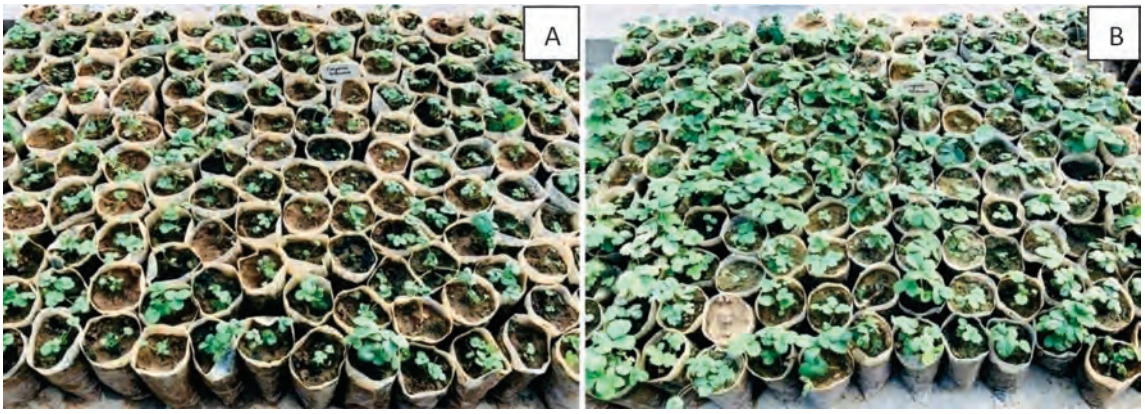

Fig. 2. Survival of micropropagated strawberry *cv*. Chandler plants in different potting mixtures. A: Plants in field soil B: Plants in soil collected from chickpea field

Table 4. Percentage survival of Chandler plants in two different types of potting mixtures during hardening under screen house conditions

No. of plantlets transferred		No. of plants survived	Plant survival (%)
In field soil:	120	84	70
In leguminous soil collected from chickpea field:	121	109	90

only 70 % survival (Fig. 2; Table 4); higher frequency of plant survival in leguminous soil was most probably due to presence of increased level of nitrogen (Herridge *et al*., 1995). Kaur and Chopra (2004) reported maximum survival of plantlets (71.50 %) grown in soil and farmyard manure mixture (1:1). Haddadi *et al*. (2010) documented 90% strawberry plantlet survival in potting mixture comprising of perlite, vermiculite and cocopeat in 2:1:2 ratio.

In conclusion, during micropropagation of strawberry from nodal segments in the present study, addition of Coconut water in the culture medium was found to exert positive effect on shoot multiplication rate and overall morphology of *in vitro* regenerated plants. So, the use of Coconut water on a routine basis in shoot sub-culturing medium of strawberry is recommended. Further, MS + BAP (0.2 mg/L) + IAA (0.2 mg/L) + Kin (0.3 mg/L) + Table sugar / Sucrose (30 g/L) + Coconut water (150 ml/L) + Agar (8 g/L) medium may serve as a single step medium for explant establishment, shoot multiplication and root formation in strawberry.

Authors' contribution

Conceptualization of research work and designing of experiments (A K); Execution of field/lab experiments and data collection (SG, TS, JK); Analysis of data and interpretation (JK, AK, SG, TS); Preparation of manuscript (JK, SG, A K)

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