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Research article

Effect of 1-naphthalene acetic acid and 6-benzyladenine on micropropagation of strawberry cultivar 'Praratchatan No.80'

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Abstract

The growth was studied of callus, shoots and roots of the strawberry cultivar 'Praratchatan No.80' in a sterilized culture on Murashige and Skoog (MS) medium supplemented with different concentrations and combinations of 1-naphthalene acetic acid (NAA) and 6-benzyladenine (BA). Treatments were established as MS medium supplemented with combinations of NAA (0.0 mg/L, 1.0 mg/L, 2.0 mg/L) and BA (0.0 mg/L, 1.0 mg/L, 1.5 mg/L, 2.0 mg/L) with a control (MS medium without supplementation) in a completely randomized design experiment. The results showed that MS medium supplemented with 1.0 mg/L NAA + 1.5 mg/L BA was the best medium to induce callus tissue with a maximum length and width of 2.74 cm and 2.10 cm, respectively. The callus grown on MS medium supplemented with 2.0 mg/L BA generated explants with the highest number of shoots (38.9 shoots/explant). The greatest average length of strawberry shoot was 7.24 cm. The explants from MS supplemented with 2.0 mg/L BA grown on MS without NAA generated the highest numbers of roots (16.9 roots/shoot) and root length (4.31 cm). This formula is a suitable medium for micropropagation of the strawberry cultivar 'Praratchatan No.80'.

Introduction

Cultivated strawberry (*Fragaria* × *ananassa* Duch.) is a natural hybrid of *Fragaria chiloensis* Duch. and *Fragaria virginiana* Duch. (Sakila et al., 2007). It is a herbaceous and stoloniferous perennial plant which spreads via stolons or runners (Karim et al., 2011). Strawberry plants are classified in the genus *Fragaria*, under the *Potentilleae* of the *Rosaceae* family (Naruhashi and Iwata, 1988). Some species are diploid having two sets of chromosomes (2n = 2*x* = 14), others are tetraploid (2n = 4*x* = 28), hexaploid (2n = 6*x* = 42), or decaploid (2n = 10*x* = 70) (Darnell et al., 2003) and modern cultivated strawberries are generally octoploid (2n = 8*x* = 56) (Darrow, 1966). Currently, strawberries are one of the most popular fruits in the world (Biswas et al., 2007) because of their flavor and fragrance and furthermore, they are rich in natural antioxidants that may play an important role in human health protection (Wilhelm and Sagen, 1974; Wang and Jiao, 2000; Hannum, 2004; Giampieri et al., 2012). The strawberry is also known for its digestive and tonic properties and is an important source of folate, vitamin C, fiber, potassium, flavonoids, phytochemicals, carotenoids and antioxidants (Larson, 1988; ElKichaoui, 2014). Strawberries are grown in geographically diverse areas ranging from the low-altitude tropical to subtropical to high-altitude continental areas (Darrow, 1966). In Thailand, cultivated strawberries are widely grown in the northern provinces (Chiang Mai, Chiang Rai and

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Phetchabun) where there are low temperatures and the most prominent production area in this region is Samoeng district (3,600 ha) in Chiang Mai province (Pipattanawong et al., 2011). Farmers normally begin to plant strawberries in September–November, and the harvesting period is December–April in the following year. At present, Thai strawberry growers produce approximately 10,000 Mg of fruit per year, valued at approximately USD 19 million/year (Pipattanawong et al., 2011). In addition to domestic marketing, producers export strawberries to countries such as Japan, China and Australia (Srimongkol et al., 2004) and some strawberries are also processed into jams, syrups, desserts, juices, wines, and other products (Pipattanawong, 2000).

Normally, strawberries prefer a subtropical temperature; therefore, Thai farmers have developed new cultivars that thrive in the tropical zone. Some improved cultivars which are well adapted to the Thailand's environment have been introduced to farmers with the most popular being the cultivar 'Praratchatan No.80' which has a conic to globose-conic berry shape with an average weight of 12–15 g per fruit, firmness up to 1.21 kg/cm^2 and an average sweetness of 12.85 °Brix (Pipattanawong et al., 2011). This cultivar has a good aroma, redness and firmness which make it suitable for fresh consumption after ripening (Pipattanawong et al., 2011). Due to its demand, large-scale plant propagation of this cultivar is required to increase fruit production and to meet the consumer demand. However, most farmers propagate this strawberry cultivar using runners which take too long to produce and then yield low quantities of surviving plantlets (Pipattanawong, 2000). The conventional propagation of strawberry is also faced with infection by certain diseases and insect damage (Pipattanawong, 2000). Unsanitary strawberry plantlets can cause diminished quality, low value and unacceptable marketability. Farmers have attempted to compensate for these deficiencies by using fertilizers and pesticides; however, these interventions have not been very effective and add cost to production (Pipattanawong, 2000). Previous studies have highlighted the efficiency of *in vitro* protocols for the production of true-to-type certified vegetative material for the genetic improvement of berries. These studies included tissue culture techniques to improve high quality production of strawberries that later exhibited the remarkable ability to regenerate new organs under *in vitro* conditions (Pulianmackal et al., 2014; Cappelletti et al., 2016). Various types of organs, especially apical meristem, have been successfully used for induction of new plant development (Morel and Matin, 1955; Walkey, 1978; Bhojwani and Razdan, 1983; Biswas et al., 2007).

The role of exogenous hormones is the most important factor in the development of an *in vitro* regeneration protocol (Azad et al., 2004; Subotic et al., 2009; Cappelletti et al., 2016). Recent studies have reported that the successful growth of callus shoots and root formation has been greatly influenced by different combinations and concentrations of plant growth regulators (Christianson and Warnick, 1985; Biswas et al., 2010; Su et al., 2011; Cappelletti et al., 2016). A cytokinin (6-benzyladenine; BA) and an auxin (1-naphthalene acetic acid; NAA) are the most commonly used hormones to promote callus shoot and root induction of strawberry (Letham, 1974; Akiyoshi et al., 1983; Popescu et al., 1997; Biswas et al., 2010; Koskela et al., 2012; Mouhu et al., 2013; Zhang et al., 2014). The hormone auxin was found to affect plant growth, morphogenesis and natural auxins in higher plants, where this hormone regulates cell elongation, division, differentiation and adventitious root formation (Dietz et al., 1990; Damiano et al., 2007; Biswas et al., 2010) while the cytokinin promotes cell enlargement in certain tissues (Rayle et al., 1982; Ross and Rayle, 1982; Biswas et al., 2010). Thus, in tissue culture intervention, the modification and addition of these nutrients has been carefully studied to produce strawberry plants which are disease-free, have vigorous plantlets and produce high-yielding and quality fruit, resulting in increased revenues for farmers (López-Aranda et al., 1994; George, 1996; Pipattanawong, 2000). Moreover, specific formulation of media is required for the growth promotion of these new cultivars. The objectives of this research were to study the effect of different sodium hypochlorite concentrations on the survival rates of strawberry apical meristems and the effect of growth regulators (NAA and BA) on the induction of callus, shoots and roots.

Materials and Methods

Source of strawberry and cultivation process

Strawberry runners of 'Praratchatan No. 80' were collected during winter (November–December) from Khaokho Research Station, Phetchabun province, Thailand. They were grown under greenhouse conditions at the Faculty of Agriculture, Naresuan University, Phitsanulok province, Thailand. Highly vigorous plants with high-quality fruit production without insect and disease infection were selected as stock samples for tissue culture. Runner tips of approximately 2 cm length were excised and the small leaves of the runner tips were trimmed off and gently washed in tap water for 45 min to remove surface contaminants. The prepared runner tips were dipped in different concentrations of sodium hypochlorite (NaOCl; 0%, 1%, 2%, 3%, 4%) for 5 min, 10 min, 15 min, 20 min and 25 min and then rinsed four times in sterile distilled water for 5 min each time. The runner tips were further trimmed to remove excess tissue and apical meristems and excised using aseptic conditions under a stereo microscope. These explants of apical meristem (0.5 mm length) were then cultured on Murashige and Skoog (MS) medium (Murashige and Skoog, 1962) supplemented with 30 g/L sucrose, 8 g/L agar and with a range of different growth regulator combinations of NAA and BA. The pH was adjusted to 5.8 before adding the agar and the culture media were autoclaved at 121°C for 15 min. The explants in separate culture bottles (226.80 g) were incubated in a growth chamber under 16:8 h light:darkness cycles at 25±2°C and under cool-white fluorescent lights (40 µmol/m^{2}s). The cultures were changed to new fresh media every 3 wk. These cultures were used for stock culture in the next experiment.

Induction of callus, shoots, and roots

The apical meristems of strawberry samples were induced for callus with MS supplemented with NAA and BA. The concentration combinations of NAA and BA all in milligrams per liter were 0.0+0.0, 0.0+1.0, 0.0+1.5, 0.0+2.0, 1.0+0.0, 1.0+1.0, 1.0+1.5, 1.0+2.0, 2.0+0.0, 2.0+1.0, 2.0+1.5 and 2.0+2.0 mg/L, respectively. After the microshoots were generated, they were induced using MS medium supplemented with four different concentrations of BA (0.0 mg/L, 1.0 mg/L, 1.5 mg/L, 2.0 mg/L). Micro-shoots were subsequently regenerated to be roots in MS medium supplemented with six different concentrations of NAA (0.0 mg/L, 0.1 mg/L, 0.2 mg/L, 0.3 mg/L, 0.4 mg/L, 0.5 mg/L).

The width and length of calli were measured every 7 d until 42 d. The numbers and lengths of strawberry shoots and roots were determined. The shoots were determined from 42 d to 140 d while roots were determined from 7 d to 56 d.

Transplantation of tissue culture plants to the soil

Regenerated plants, with strong stems and three leaves and a healthy root system, were selected and thoroughly washed with distilled water to remove any trace of agar and dipped in Metalaxyl (Chiatai Co., Ltd, Thailand) for 10 min before transferring into plastic bags containing a 1:1 (volume per volume; v/v) mixture of sterile garden soil and sand. Plants were maintained in a humidity chamber for 2 wk and then planted in the field. The strawberry plantlets were photographed using a digital camera.

Experimental design and statistical analysis

A completely randomized design was used for all experiments. Treatments for induction of shoots and roots consisted of 20 replications, while treatments for induction of callus used 15 replications. Univariate analysis ($p < 0.05$) of the data used the SPSS program version 17.0 software (SPSS Inc; Chicago, IL, USA). Significance levels among treatment means were analyzed using Duncan's multiple range test.

Results

Effect of different sodium hypochlorite concentrations on survival rates of strawberry apical meristems

Strawberry apical meristems that were subjected to five different sterilization treatments using various NaOCl concentrations had the highest survival rate (80%) when they had been treated with 3% NaOCl for 20 min, whereas only a 40% survival rate resulted from treatment with 2% NaOCl for the same duration. Longer treatment periods (an additional 5 min) resulted in significantly lower survival rates. (Fig. 1).

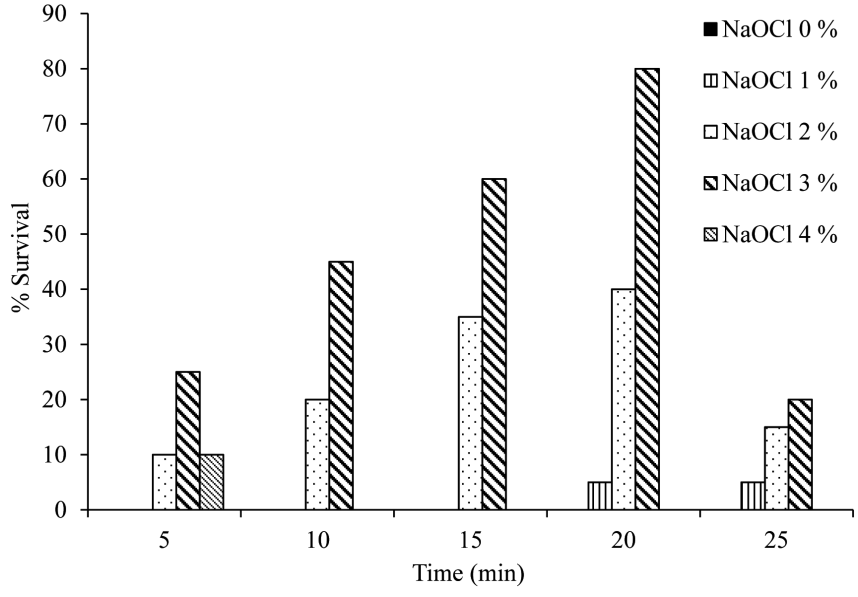

Fig. 1 Survival of apical meristems of strawberry affected by four different concentrations of sodium hypochlorite for different times of immersion, where immersion in water was used as the control treatment (0% sodium hypochlorite); % survival was calculated from number of explants that survived browning and contamination after 14 d.

Effect of growth regulators on induction of callus, shoots, and roots

The optimal medium formulation to induce the largest size of callus consisted of 1.0 mg/L NAA + 1.5 mg/L BA (Tables 1, 2 and Fig. 4A). The widths and lengths of callus under these conditions were significantly different. In contrast, the medium supplemented with only BA (0.0 mg/L NAA + 1.0 mg/L BA, 0.0 mg/L NAA + 1.5 mg/L BA and 0.0 mg/L NAA + 2.0 mg/L BA) did not induce callus (Tables 1 and 2). Calli grown using the optimized callus induction medium (1.0 mg/L NAA + 1.5 mg/L BA) were then subjected to shoot induction in four treatments containing different BA concentrations (0.0 mg/L, 1.0 mg/L, 1.5 mg/L, 2.0 mg/L) and the highest shoot number and the greatest shoot length (38.9 shoots/explant and 7.24 cm, respectively) were observed using 2.0 mg/L BA after 140 d. Both these results (number and shoot length) were higher than those among other treatments ($p < 0.05$). Overall, the shoot numbers and shoot length increased as the concentration of BA increased (Figs. 2A and 2B).

The shoots from the best medium were transferred into MS media supplemented with six different NAA concentrations (0.0 mg/L, 0.1 mg/L, 0.2 mg/L, 0.3 mg/L, 0.4 mg/L, 0.5 mg/L) for root induction. After 56 d of observation, MS without NAA produced the highest number of and the longest roots (16.9 roots/shoot and 4.31 cm, respectively; Figs. 3A and 3B).

Table 1 Effect of plant growth regulators on callus width (centimeters) in the strawberry cultivar 'Praratchatan No.80'.

NAA+BA concentration (mg/L)	Time in culture						Degree of callus development
	7 d	14 d	21 d	28 d	35 d	42 d	
0.0+0.0	0±0^{a}	0.62±0.18^{bcd}	0.74±0.30^{cd}	1.00±0.35^{c}	1.20±0.53^{b}	1.21±0.53^{b}	++
0.0+1.0	0±0^{a}	0±0^{f}	0±0^{f}	0±0^{d}	0±0^{c}	0±0^{c}	–
0.0+1.5	0±0^{a}	0±0^{f}	0±0^{f}	0±0^{d}	0±0^{c}	0±0^{c}	–
0.0+2.0	0±0^{a}	0±0^{f}	0±0^{f}	0±0^{d}	0±0^{c}	0±0^{c}	–
1.0+0.0	0±0^{a}	0.42±0.12^{e}	0.54±0.15^{e}	1.12±0.24^{bc}	1.29±0.35^{b}	1.35±0.48^{b}	++
1.0+1.0	0±0^{a}	0.49±0.16^{de}	0.64±0.17^{de}	1.06±0.18^{bc}	1.30±0.30^{b}	1.54±0.61^{b}	++
1.0+1.5	0±0^{a}	0.92±0.20^{a}	1.14±0.28^{a}	1.41±0.42^{a}	1.79±0.53^{a}	2.10±0.77^{a}	+++
1.0+2.0	0±0^{a}	0.66±0.27^{bc}	1.04±0.31^{ab}	1.22±0.37^{ab}	1.70±0.76^{a}	1.96±1.01^{a}	++
2.0+0.0	0±0^{a}	0.53±0.16^{cde}	0.75±0.22^{cd}	1.25±0.46^{ab}	1.36±0.51^{b}	1.50±0.72^{b}	++
2.0+1.0	0±0^{a}	0.48±0.25^{de}	0.88±0.14^{c}	0.98±0.28^{c}	1.33±0.32^{b}	1.54±0.45^{b}	++
2.0+1.5	0±0^{a}	0.73±0.19^{b}	1.08±0.25^{a}	1.24±0.36^{ab}	1.70±0.49^{a}	2.20±0.64^{a}	+++
2.0+2.0	0±0^{a}	0.54±0.33^{cde}	0.90±0.32^{bc}	0.94±0.24^{c}	1.22±0.52^{b}	1.44±0.76^{b}	++

mean values (±SD) with different lowercase superscripts within a column are significantly different ($p < 0.05$).
+ = little callusing (0–1 cm); ++ = moderate callusing (1.1–2 cm); +++ = high callusing (2.1–3 cm).

Table 2 Effect of plant growth regulators on length of callus (centimeters) in the strawberry cultivar 'Praratchatan No.80'.

NAA+BA concentration (mg/L)	Time in culture						Degree of callus development
	7 d	14 d	21 d	28 d	35 d	42 d	
0.0+0.0	0±0^{a}	1.12±0.25^{cd}	1.32±0.37^{bc}	1.42±0.49^{b}	1.55±0.66^{b}	1.60±0.68^{c}	++
0.0+1.0	0±0^{a}	0±0^{e}	0±0^{d}	0±0^{c}	0±0^{c}	0±0^{d}	–
0.0+1.5	0±0^{a}	0±0^{e}	0±0^{d}	0±0^{c}	0±0^{c}	0±0^{d}	–
0.0+2.0	0±0^{a}	0±0^{e}	0±0^{d}	0±0^{c}	0±0^{c}	0±0^{d}	–
1.0+0.0	0±0^{a}	1.05±0.17^{cd}	1.18±0.15^{c}	1.34±0.37^{b}	1.49±0.45^{b}	1.65±0.56^{c}	++
1.0+1.0	0±0^{a}	1.18±0.23^{abc}	1.30±0.25^{bc}	1.42±0.33^{b}	1.70±0.42^{b}	2.16±0.86^{bc}	+++
1.0+1.5	0±0^{a}	1.20±0.22^{abc}	1.58±0.43^{a}	1.82±0.53^{a}	2.40±0.72^{a}	2.74±0.94^{a}	+++
1.0+2.0	0±0^{a}	1.29±0.29^{ab}	1.52±0.42^{ab}	1.77±0.62^{a}	2.23±1.00^{a}	2.54±1.28^{ab}	+++
2.0+0.0	0±0^{a}	1.15±0.19^{bcd}	1.37±0.29^{abc}	1.58±0.41^{ab}	1.72±0.54^{b}	1.98±0.75^{bc}	++
2.0+1.0	0±0^{a}	1.02±0.13^{d}	1.26±0.26^{c}	1.40±0.27^{b}	1.77±0.56^{b}	2.08±0.67^{bc}	++
2.0+1.5	0±0^{a}	1.32±0.28^{a}	1.54±0.39^{ab}	1.86±0.39^{a}	2.27±0.59^{a}	2.90±0.89^{a}	+++
2.0+2.0	0±0^{a}	1.08±0.35^{cd}	1.24±0.43^{c}	1.38±0.50^{b}	1.68±0.79^{b}	2.01±1.14^{bc}	++

mean values (±SD) with different lowercase superscripts within a column are significantly different ($p < 0.05$).
+ = little callusing (0–1 cm); ++ = moderate callusing (1.1–2 cm); +++ = high callusing (2.1–3 cm).

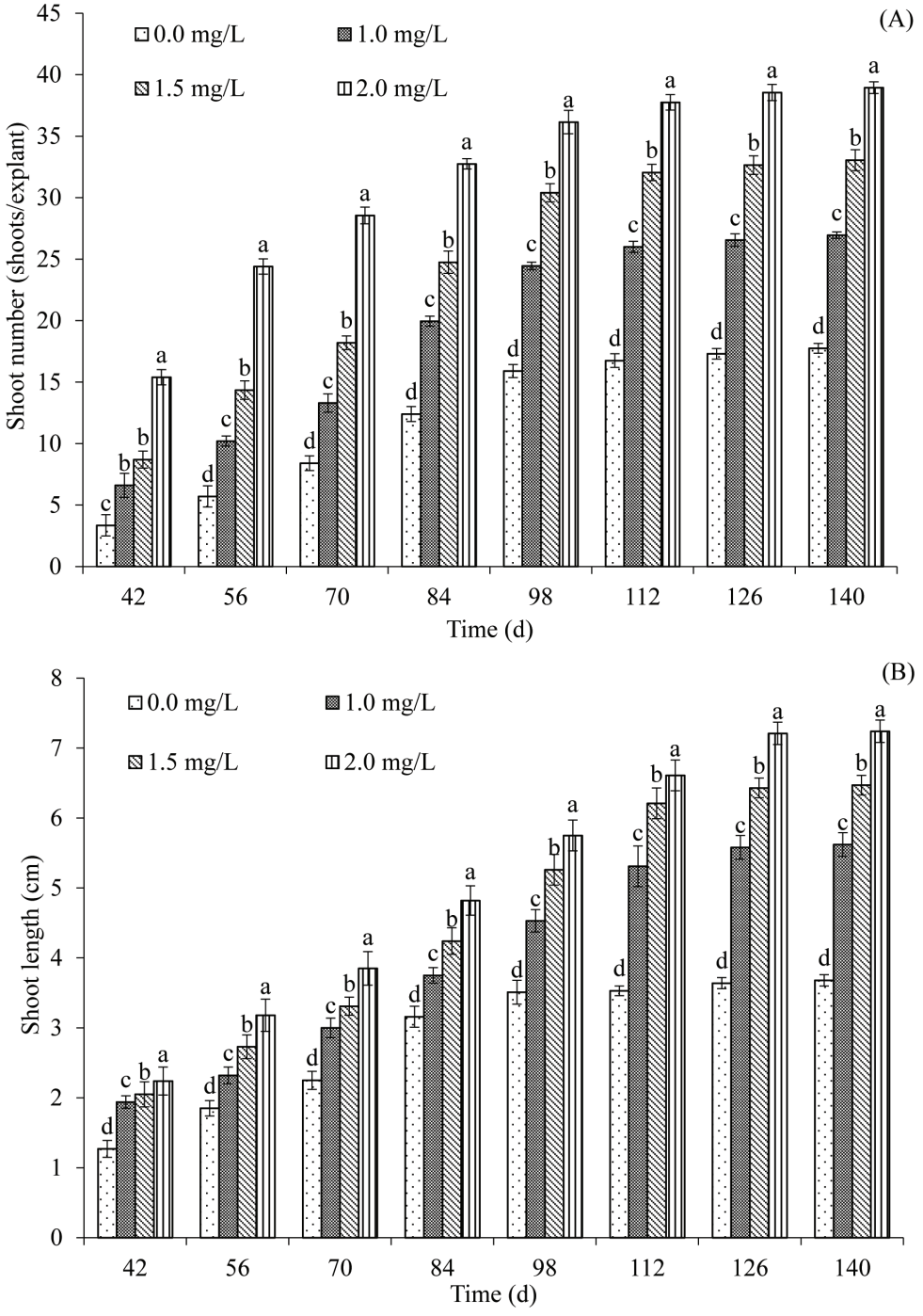

Fig. 2 Effect of the growth regulator 6-benzyladenine on induction of strawberry cultivar 'Praratchatan No.80': (A) shoot number; (B) shoot length, where error bar = ± SD, $n = 20$ and different lowercase letters above bars indicate significant differences at each cultivation time (d) ($p < 0.05$)

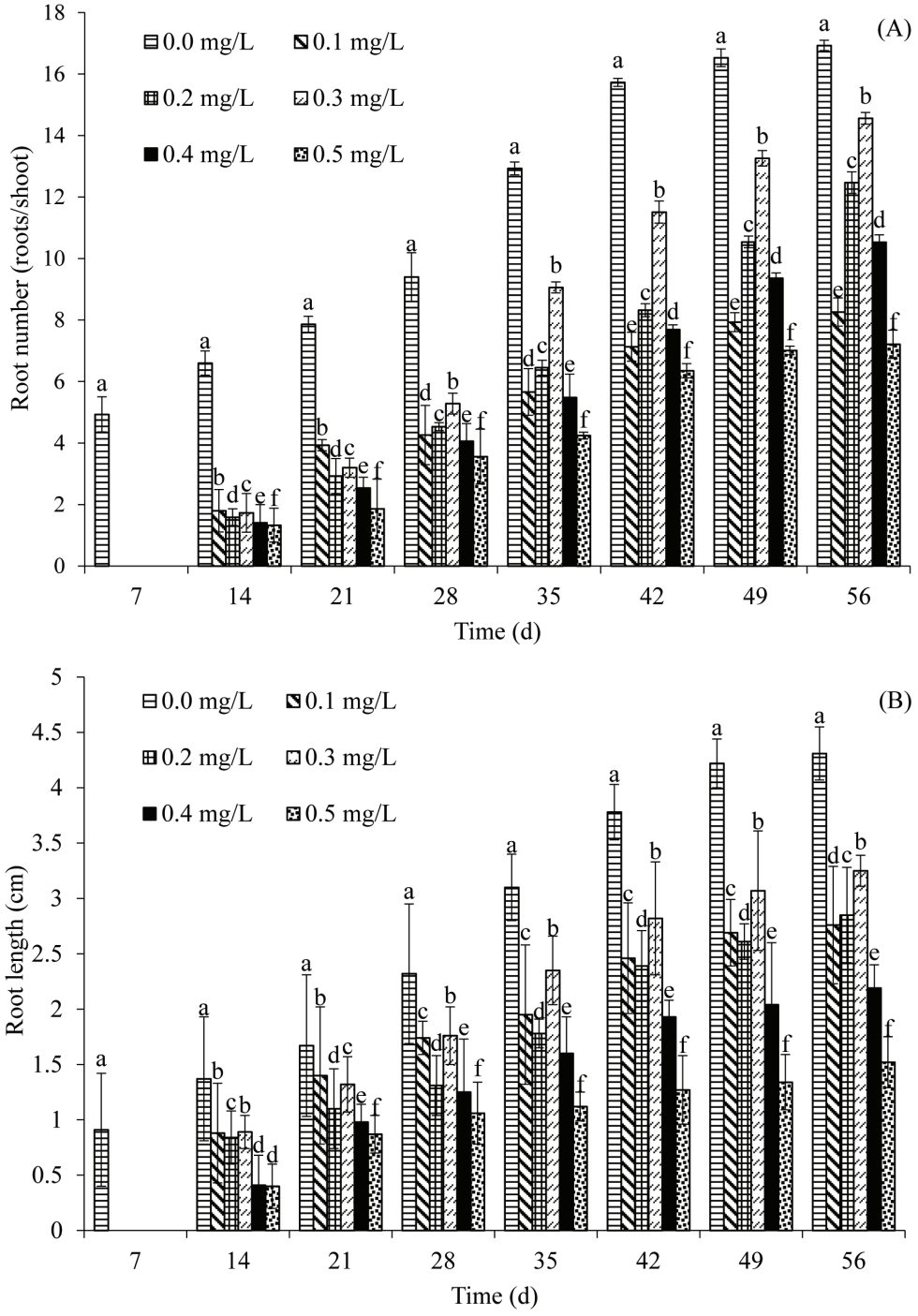

Fig. 3 Effect of the growth regulator 1-naphthalene acetic acid on the induction of strawberry cultivar 'Praratchatan No.80': (A) root number; (B) root length, where error bar = ± SD, $n = 20$ and different lowercase letters above bars indicate significant differences at each cultivation time (d) ($p < 0.05$)

The morphological characteristics associated with survival and the vigorous plantlets of strawberry at 84 d and 112 d in MS medium supplemented with 2 mg/L of BA are shown in Figs. 4B and 4C, respectively. The morphological characteristics of these plantlet roots are shown in Fig. 4D. The characteristics of vigorous strawberry cultivars of 'Praratchatan No.80' after transferring from the medium into sterile garden soil and sand (1:1 v/v) for 1 mth are shown in Figs. 4E and 4F.

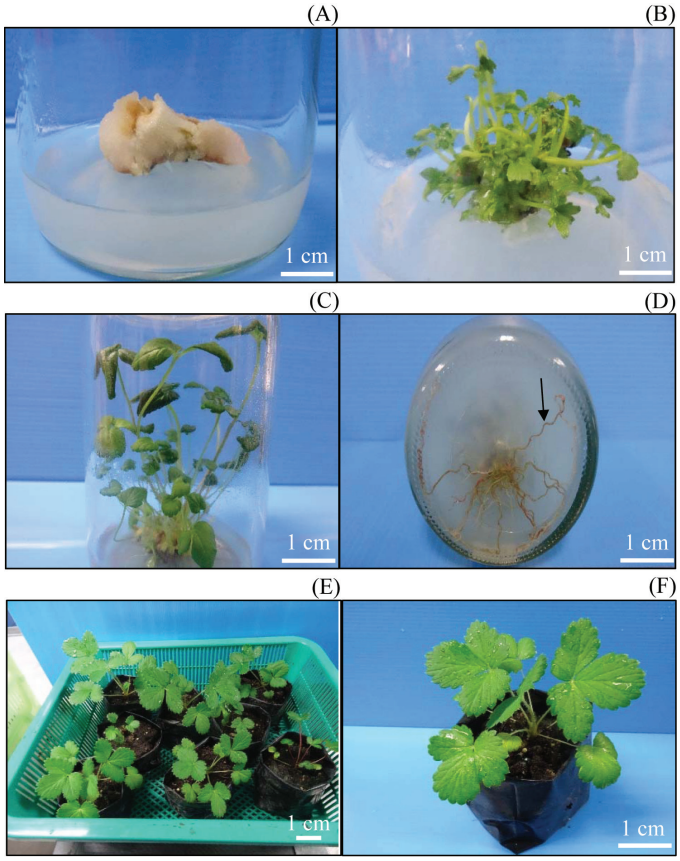

Fig. 4 Strawberry micropropagation from runner tips: (A) callus induction on Murashige and Skoog (MS) medium supplemented with 1.0 mg/L 1-naphthalene acetic acid (NAA) + 1.5 mg/L 6-benzyladenine (BA); (B–C) shoot proliferation on MS supplemented with 2 mg/L BA after 84 d and after 112 d of culture, respectively; (D) root formation stage on MS without NAA after 56 d; (E–F) acclimatization stage under greenhouse conditions of tissue cultured strawberry for 1 mth.

Discussion

The results showed that treating with 3% sodium hypochlorite for 20 min resulted in the maximum number of aseptic cultures and a higher survival rate of apical meristems than using 2% sodium hypochlorite ($p < 0.05$). The apical meristems of strawberry may be resistant to 3% sodium hypochlorite while at the same time, this concentration of sodium hypochlorite can effectively destroy the microbial contamination. This result agreed with findings for optimal surface sterilization of strawberry explants of runner tips using 2.5% sodium hypochlorite for 20 min (ElKichaoui, 2014), of runner tips using 3.75% commercial bleach (sodium hypochlorite) for 15 min (Danial et al. 2016) and of nodal segments using commercial bleach (3.5% commercial bleach with the sodium hypochlorite concentration <5%) for 20 min (Cappelletti et al. 2016). The significantly largest size of callus was induced using MS medium consisting of 1.0 mg/L NAA + 1.5 mg/L BA. Similar results were reported with the same medium inducing callus formation from three sources (leaf blade, nodal, runner segments) of strawberry explants (Biswas et al., 2010). The results were contrasted with the results of Karim et al. (2015) who demonstrated that 2.0 mg/L NAA + 0.5 mg/L BA could induce the maximum callus formation from *in vitro*-grown strawberry leaf explants. In addition, somaclonal variation had a low probability of occurring since shoot-derived callus regeneration uses organogenesis and only one subculture was used in the current study. This confirmed the results of Biswas et al. (2009) that somatic embryogenesis had the highest percentage of somaclonal variation using 12 subcultures. It has also been reported that the number of subcultures (3–8) did not affect the productivity but problems arose when using material with over 10 subcultures (López-Aranda et al., 1994). Moreover, similar findings were reported by Sen and Dhawan (2010) who stated that tissue culture-raised plantlets of strawberry could change their genetic and clonal uniformity when over 15 subcultures were used.

The current results also showed that media supplemented with only BA (0.0 mg/L NAA + 1.0 mg/L BA, 0.0 mg/L NAA + 1.5 mg/L BA, 0.0 mg/L NAA + 2.0 mg/L BA) could not induce callus (Tables 1 and 2). This was expected, since it had been shown previously that cytokinins (BA) promote cell enlargement in certain tissues and have been used in micropropagation media to stimulate shoot multiplication (Boxus, 1974). The interactions between the plant auxins and the cytokinins throughout plant development are complex, and the balance between the auxins and cytokinins controls the formation of roots, shoots and callus tissue *in vitro* (Landi and Mezzetti, 2006). The present investigation found that auxin combined with cytokinin (NAA+BA) was the most effective for callus induction as did Biswas et al. (2010) and Karim et al. (2011). In addition, many factors such as genotype (Liu and Sanford, 1988; Passey et al., 2003), type and source of explant (Biswas et al., 2010; Kumar and Reddy, 2011), nutrient medium and physical growth factors such as light, temperature, humidity, and endogenous supply of growth regulators are important for callus induction (Pierik, 1987)

The MS medium supplemented with 2.0 mg/L BA was the most effective for shoot induction ($p < 0.05$). These results agreed with those of Ara et al. (2012) who demonstrated that 2.0 mg/L 6-benzylaminopurine (BAP) was the best medium for generating shoot formation in the cultivar 'JP-2' (40% frequency of regeneration, 5.3 shoots regenerated). In addition, Danial et al. (2016) reported that 2.0 mg/L BAP was the best medium for generating shoot strawberry formation with *in vitro* propagation in the Kurdistan region of Iraq (3.16 shoots regenerated/explant). The results contrasted with Zobayer et al. (2011) who reported that 2.0 mg/L BA could not induce shoot formation in the 'RABI Strawberry-3' cultivar, while Boxus (1999) reported that shoots proliferated when shoots tips of strawberry were cultured on a medium supplemented with 0.5 mg/L BA and

6.4 g/L agar. In addition, Marcotrigiano et al. (1984) found that BA at 0.3 mg/L effectively stimulated shoot proliferation at 1.0 mg/L and 3.0 mg/L. Simpson and Bell (1989) found that a BA level of 1.0 mg/L produced the best results for the cultivar Sweet Charlie. Cappelletti et al. (2016) and reported that 3.0 mg/L BA was the best medium for generating the highest number of shoots per plant in the cultivar 'selva' (10.3 shoots per leaf).The results of these various studies may imply that different strawberry varieties respond differently to different concentrations and combinations of plant growth regulators (Passey et al., 2003) and even different organs of the same plant are characterized not only by their unique intrinsic biochemical changes but also by the sensitivity of the endogenously supplied chemical stimuli (Youssief, 2009). Moreover, MS medium without NAA was the most effective medium for root induction of strawberry. Similar results were also observed by Ara et al. (2012) where root induction (100%) was performed using regenerated shoots of the strawberry cultivar 'JP-2' that were sub-cultured on MS media without any growth regulators. These results also agreed with the findings of ElKichaoui (2014) who reported that full MS without plant growth regulators could produce the highest root number, root length and shoot elongation. Similar results were also reported by Emarah (2008) and Youssief (2009) who highlighted the effect of MS strengths on root length, revealing that the greatest length was obtained with full MS without any intervention. In the current experiment, using full-strength MS media without plant growth regulators for the establishment of the rooting stage of strawberry provided significantly optimal results. This may imply that there are hormones, nutrients and other chemicals present in strawberries which may play an important role in root stimulation. Hence, this would be an ideal topic for future investigations.

Conflict of Interest

The authors declare that there are no conflicts of interest.

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