Journal of Applied Horticulture, 17(3): 192-198, 2015

In vitro explant sterilization and bud initiation studies of four strawberry cultivars

M. Munir[1]*, S. Iqbal[2], J.U.D. Baloch[2] and A.A. Khakwani[2]

*[1]Fronteir Agriculture, SOYL Precision Crop Production Division, Newbury, RG14 5PX, United Kingdom. [2]Faculty of Agriculture, Gomal University, Dera Ismail Khan, KPK, Pakistan. *E-mail: muhammad.munir@soyl.co.uk*

Abstract

A series of *in vitro* experiments were conducted using four strawberry cultivars to investigate their survival response to different disinfectants, explants regeneration response to liquid and solid media, *in vitro* bud initiation response to BAP enriched media, clonal multiplication response to various BAP concentrations in MS and Knop media and bud initiation response to sugars sources. Highest meristem survival (75%) was recorded in cultivars Osogrande and Toro when treated with 0.5% NaOCl for 15 minutes, however, 75% explants of Chandler survived when treated with 1% NaOCl for 10 minutes. Similarly, maximum survival (58-71%) was observed in Chandler, Osogrande and Islamabad Local when internodal segments were treated with 0.5% NaOCl for 15 minutes. However, the survival percentage of these cultivars significantly varied at different NaOCl concentrations when petioles segments were used as explants. The results of second experiment indicated that highest rate of survival (79.20%) was achieved in Toro when meristems were cultured on solid MS media containing 0.5 mg/L GA_3. In another experiment, maximum percentage (83) of bud initiation was recorded in Osogrande at 0.5 mg/L BAP. Findings of experiment regarding clonal multiplication of *in vitro* shoots derived from meristem showed that maximum buds formation per culture (25, 20 and 15) were obtained in MS media containing 1.5 mg/L BAP and 0.1 mg/L IBA in cultivars Osogrande, Chandler and Islamabad Local respectively, however, similar buds formation response was varied with the cultivars when knop media was used. Similarly, when sugar sources were studied cultivar Osogrande initiated highest number of buds (20) at sucrose based MS media containing 0.8 mg/L Kinetin and 0.2 mg/L NAA, however, cultivars Chandler and Islamabad Local initiated 15 buds at sucrose based MS media containing 0.6 mg/L Kinetin and 0.2 mg/L NAA.

Key words: Strawberry, *Fragaria* × *ananassa*, explants sterilization, *in vitro* bud initiation, media formulation

Introduction

Strawberry (*Fragaria* × *ananassa* Duch.) is commonly propagated by runners (restricted to particular season) and like other vegetatively propagated plants it is often infected by pathogens, resulting in low yield (Martin and Tzanetakis, 2006). In field propagation it is important to use healthy plants, however, it is not possible through traditional methods of propagation. Moreover, propagation of strawberries through runners does not fulfill the huge market demand as it is cultivated on 241109 ha in the world and produced 4516810 tonnes of fruits. Asia contributes only 13% of the world strawberries cultivated area and produces only 18% of the world total strawberries production (Food and Agriculture Organization, 2012). Therefore, there is a need to look into the new propagation techniques for this crop to increase area and production.

During the last three decades it has become possible to regenerate plantlets *in vitro* from plant parts or tissues. *In vitro* plant tissue culture technique is used for the production of pharmaceuticals and other natural products, the genetic improvement of crops, the recovery of disease-free clones and preservation of valuable germplasm and rapid clonal multiplication of promising varieties. Plants raised through this technique require aseptic growing environment, however, contamination frequently originates with the introduction of explants into the culture contaminated with endophytic micro-organisms or surface sterilization resistant micro-organisms. Heterotrophic plant tissue media are capable of supporting the growth of many common environmental micro-organisms which may provide a food source for micro-arthropods and can act as vectors in the spread of laboratory contamination. Growing of *in vitro* produced contaminated plants in the fields bring along the risk of large-scale disease and poor *in vivo* plant performance. The best strategy to control tissue culture contamination is to establish aseptic cultures and to maintain good laboratory practice including a viable procedure of explants sterilization (Moisander and Herrington, 2006).

Media used for *in vitro* culture contain nutritional components which are essential for growth and morphogenesis of cultured explants. The success of *in vitro* culture also depends on the media formulation, which varies with the species and genotype, and is probably a reflection of the multiplication capacity of the explants. The use of MS micro-elements, Knop macro-elements, MS vitamin mixture, glucose, and agar at pH 5.6 was recommended by Boxus (1999) for strawberry *in vitro* propagation. Similarly, Atkinson *et al*. (1986) found that composition of the *in vitro* propagation media (MS or B5) and the concentration of BA affect both the initial crop establishment and the density of crowns produced subsequently. Previous studies conducted on different strawberry cultivars mainly focused on media formulation and regeneration using plant growth regulators. However, in present studies we have selected three commercial and one local strawberry cultivars and attempted to develop a consolidated protocol from explants sterilization to plantlet regeneration along with the comparative response to the media formulation. Keeping in view these objectives a series of experiments were designed

to determine a suitable explants disinfectant technique, to assess the suitability of liquid or solid media, to compare MS and Knop media and to formulate an appropriate explants regeneration media having apposite sugar source.

Materials and methods

Choice of explants: Four commercial cultivars of strawberry (Chandler, Osogrande, Toro and Islamabad Local) were obtained from Horticultural Research Institute, National Agricultural Research Centre, Islamabad, Pakistan and from private fruit farms at Murree hills.

Experiment 1. *In vitro* explants sterilization: Explants (shoot tips, internodal segments and petioles) of field grown runners were washed with tap water for 20min. Then these explants were disinfected by dipping in 70% ethanol for 2 seconds, followed by surface sterilization with clorox bleach, 0, 1, 2, and 2.5% sodium hypochlorite (NaOCl) for 0, 5, 10 and 15min. Twenty four explants were used for each treatment. The data was recorded in terms of survival, browning, and contamination percentage.

Nutrient media: Explants were cultured on Murashige and Skoog media (1962). The pH of the media was adjusted to 5.8 prior to autoclave. The media was dispensed in 250 mL flasks capped with cotton plugs and was sterilized by using autoclave for 15 min at 121°C.

Experiment 2. Meristem culture using solid and liquid media: Meristems of 0.5-1 mm size, from the runners of all four cultivars were excised with the help of low power dissecting microscope and were cultured on both liquid and solid media. Easy and effective method for excision was noted when approach (with fine blade) was made towards meristem from distal end. Thin and transverse slices were made, until a cone including the apical dome with 1-2 embryonic leaves were teased away. Number of total meristems were 24 for each treatment. The data was recorded on dried and survival percentage of meristem. The following media was tested for survival of meristems:

Media used : Murashige and Skoog (1962)
Growth regulator : 0.5 mg/L GA_3
State of media : Liquid (filter paper bridge) and solid (agar 7 g/L)
Amount of sugar used : 21.6 g/L (sucrose)

Culture conditions: The cultures were kept in 16 h photoperiod under cool inflorescent light intensity of 2000 lux at temperature of 23±2 °C.

Experiment 3. *In vitro* bud initiation: Explants (meristem) of field grown strawberry runners (Chandler, Osogrande, Toro and Islamabad Local) were washed with tap water for 20 min. These were then disinfected by dipping in 70% ethanol for 2 seconds, followed by surface sterilization with 'clorox bleach' containing 0.5% Sodium hypochlorite for 15 min.

Experiment 3.1. Effect of BAP (0.5 mg/L) on bud initiation: Small plantlets produced through meristem culture were transferred to fresh media containing 0.5 mg/L BAP for axillary/adventitious buds initiation. Twenty plants of each cultivar were selected for the experiment and the data were collected when plants showed *in vitro* bud initiation.

Experiment 3.2. Clonal multiplication with MS and Knop media: For clonal multiplication the initiated buds (in compact form) were regularly sub-cultured on the same media composition (MS with 0.5 mg/L BAP). To determine the optimum concentration of growth regulators for clonal multiplication, the initiated buds were cultured on MS or Knop media with different concentrations of BAP (0, 0.5, 1, 1.5, 2, 2.5 and 3 mg/L) and IBA (0 and 0.1 mg/L). Sucrose (30 g/L) was used as source of sugar while 5.6 pH was maintained in the media. Initial number of buds were 2-3 per culture. The data of the number of bud initiation were recorded after 10-days of culture.

Experiment 3.3. Effect of sugar source on bud initiation: As a carbohydrate source, the effect of glucose and sucrose was studied in MS culture media. For axillary/adventitious buds initiation, a constant amount of glucose or sucrose (30 g/L) was used with different growth regulators (Kinetin 0, 0.2, 0.4, 0.6, 0.8 mg/L and NAA 0, 0.2 mg/L). Number of initial buds per culture were 2-3. The data were recorded in terms of bud initiated in culture.

All experiments were laid out on Randomized Complete Design and the data obtained were analyzed using Genstat-8 software, (Lawes Agricultural Trust, Rothamsted Experimental Station, UK). After calculating the ANOVA, least significant difference (LSD) test was applied to compare treatment group means. In order to indicate variability within sample means, standard errors within replicates were also estimated which are either shown as y-bar in graphs or presented as plus/minus symbol in tables.

Results and discussion

Experiment 1. *In vitro* explants sterilization: Maximum meristems (75%) survival of Osogrande and Toro was recorded, when treated with 0.5% NaOCl for 15 min (Fig. 1a). However, similar percentage of survival was observed in Chandler when its meristems were treated with 1% NaOCl for 10 min. Islamabad Local cultivar also showed satisfactory results, 66.7% survival at treatment of 2.5% NaOCl for 5 min. Similarly, maximum browning percentage (75) was noted in Osogrande when meristem explants were treated with 2.5% NaOCl for 10 min (Fig. 1b). However, the browning problem declined significantly ($P<0.05$) in Osogrande, Toro and Chandler when the meristems

Table 1. Murashige and Skoog media with growth regulators for axillary/adventitious bud initiation

Growth regulators (mg/L)		Number of bud initiated			
BAP	IBA	Chandler	Osogrande	Toro	Islamabad Local
0.0	0.0	3 (±0.58)	3 (±0.58)	4 (±0.58)	4 (±1.16)
0.5	0.1	10 (±1.73)	10 (±1.16)	4 (±1).16)	10 (±0.58)
1.0	0.1	15 (±1.16)	15 (±0.58)	5 (±1.16)	10 (±1.16)
1.5	0.1	20 (±1.73)	25 (±1.73)	5 (±0.58)	15 (±1.16)
2.0	0.1	15 (±1.16)	20 (±1.73)	5 (±0.58)	10 (±1.16)
2.5	0.1	10 (±1.16)	20 (±1.73)	4 (±1.16)	10 (±0.58)
3.0	0.1	10 (±1.16)	10 (±0.58)	3 (±0.58)	5 (±1.16)
$LSD_{0.05}$		4.29	3.62	0.95	3.41

Values in parenthesis are standard errors within replicates whereas as least significant differences and standard error differences among means are based on 5% level of probability.

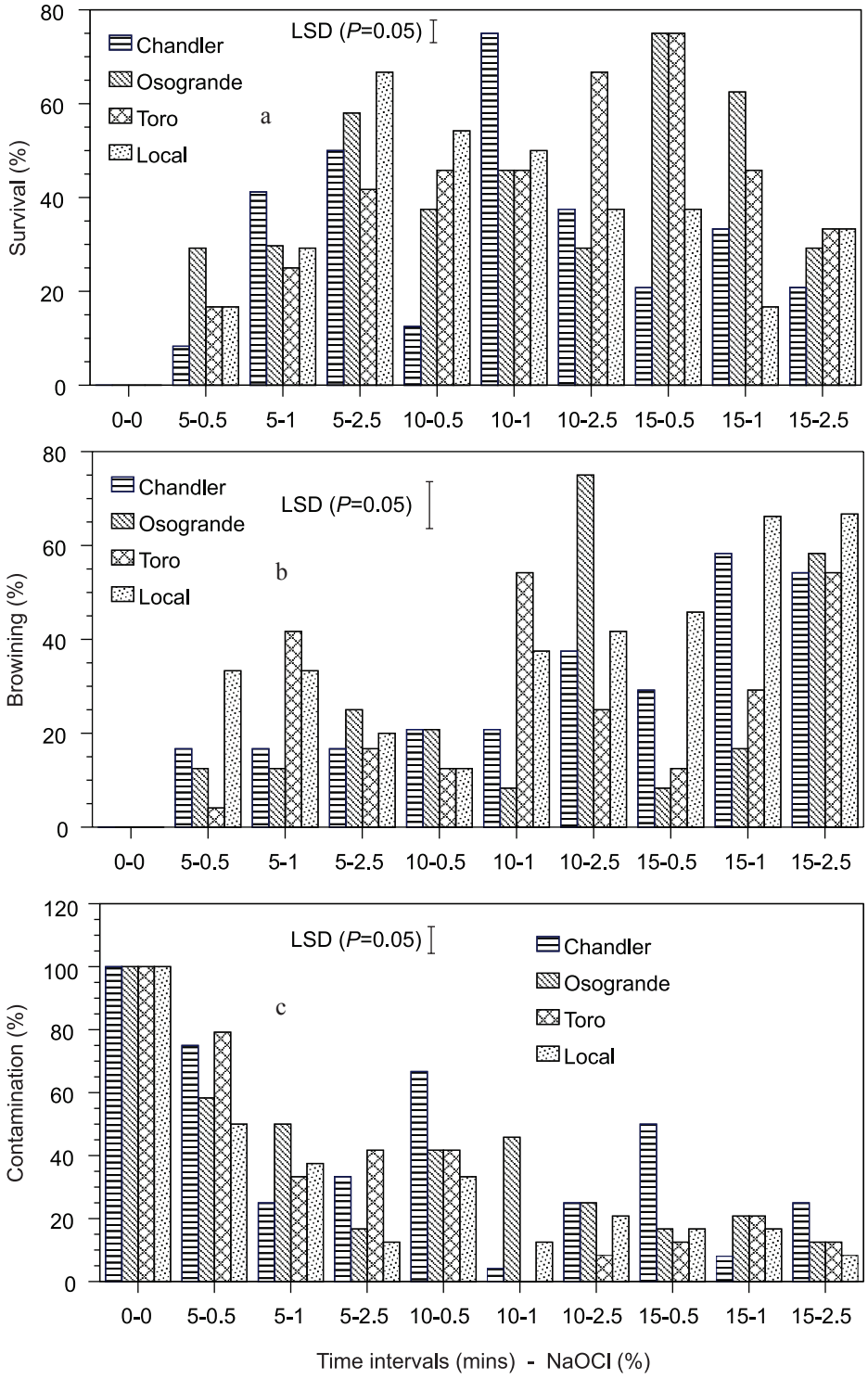

Fig. 1. Response of four strawberry cultivars *viz.,* Chandler, Osogrande, Toro and Islamabad Local to different treatments of NaOCl (0.5, 1 and 2.5%) at different time intervals (5, 10 and 15 minutes) on (a) survival, (b) browning and (c) contamination percentage of meristem explants. Vertical bars represent the least significant difference (LSD, *P*=0.05) within means.

sterilized with 0.5% NaOCl for 5min. In Islamabad Local, the similar level of NaOCl was effective but the meristems were kept in the disinfectant for 10 min. Fig. 1c showed 100% contamination of untreated meristem explants. However, meristems taken from Chandler and Toro showed minimum contamination at 1% NaOCl concentration for 10 min treatment. The other two cultivars showed similar results when the meristems were treated with 2.5% NaOCl concentration for 15 min.

Explants derived from internodal segments of cultivar Chandler showed higher survival rate (71%) when treated with 0.5% NaOCl for 15 min (Fig. 2a). However, internodal segments from Osogrande and Islamabad Local exhibited 63 and 58% survival respectively at the similar level of NaOCl treatment. Similarly, maximum browning percentage, 58% (Chandler and Islamabad Local), 62% (Osogrande) and 67% (Toro) was recorded when internodal segments were treated with 2.5% NaOCl for 15 min (Fig. 2b). 100% contamination was observed in untreated explants followed by five minutes treatment of explants with 0.5% NaOCl (Fig. 2c). However, contamination percentage decreased with the increase in time interval and the concentration of NaOCl.

Fig. 3a indicated that 63% explants taken from petiole segments survived in Islamabad Local (0.5% NaOCl for 10min) followed by Chandler (58%, 0.5% NaOCl for 15 min), Toro (50%, 1% NaOCl for 10 min) and Osogrande (38%, 1% NaOCl for 5 min). As for as browning percentage is concerned (Fig. 3b), 75% browning was observed in Osogrande followed by Islamabad Local (50%) and Chandler (42%) when petiole segments were treated with 2.5% NaOCl for 15 min. Maximum contamination percentage was again noted in untreated explants (Fig. 3c). Like previous observations, contamination percentage decreased with the increase in time interval and the concentration of NaOCl. Similar results were obtained by Seemuller and Merkle (1984) and Nehra *et al.* (1990).

Experiment 2. Meristem culture on solid and liquid media: Findings of Experiment 1 revealed that shoot tip meristem is the best explants source hence was used for further experiments. Meristems were cultured on MS media containing 0.5 mg/L GA_3 (liquid and solid) stated. It was observed that the response to culture media varied from cultivar to cultivar. It is apparent from Fig. 4ab that the best meristems survival percentage (79) was obtained using solid media for cultivar Toro. However, survival percentage for other cultivars such as Chandler (75%), Osogrande (71%) and Islamabad Local (63%) significantly declined when liquid media was used. Badawi *et al.* (1990) cultured meristems of cultivars Pajaro, Tioga, and Tufts on solid or liquid media supplemented with various concentrations of IBA, GA_3 and BA, and observed that shoot formation was generally obtained on solid media supplemented with 1 mg/L BA, 1 mg/L GA_3 and 0.2 mg/L IBA. Similarly, Biswas *et al.* (2007) cultured meristems on filter paper bridge containing test tube in liquid MS media and on solid MS media and reported that quick establishment of meristem was observed in liquid media containing 0.1 mg/L GA_3.

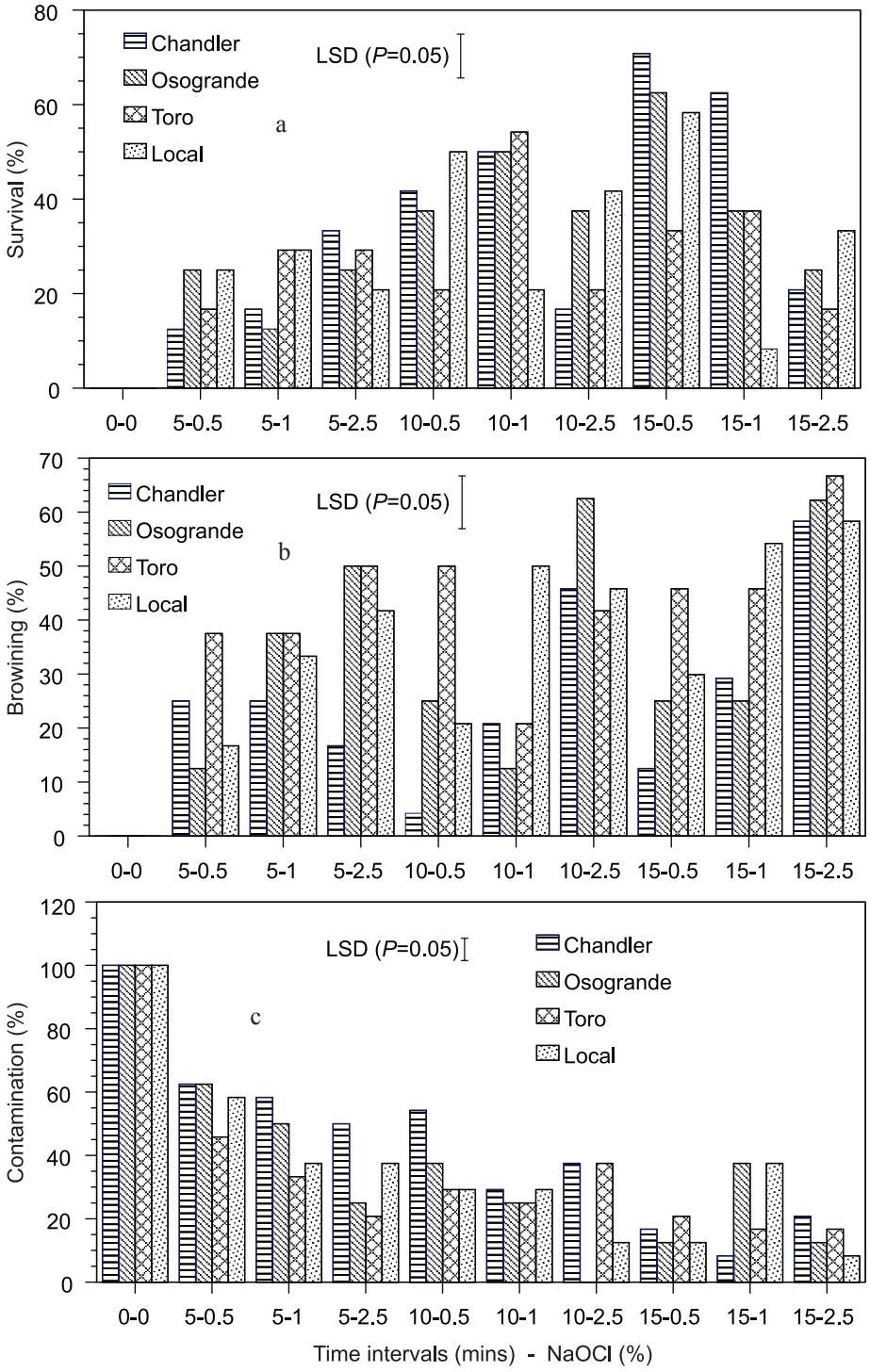

Fig. 2. Response of four strawberry cultivars *viz.,* Chandler, Osogrande, Toro and Islamabad Local to different treatments of NaOCl (0.5, 1 and 2.5%) at different time intervals (5, 10 and 15 minutes) on (a) survival, (b) browning and (c) contamination percentage of internodal segments explants. Vertical bars represent the least significant difference (LSD, *P*=0.05) within means.

Experiment 3. In vitro bud initiation

Experiment 3.1. Effect of BAP (0.5 mg/L) on bud initiation: All strawberry cultivars regenerated shoots through *in vitro* meristems cultured on MS media enriched with BAP (0.5 mg/L). Fig. 5 showed that 83% plantlets (1-2 cm size) initiated buds on the given concentration of BAP in Osogrande. Similarly, 64% (Chandler), 23% (Toro) and 46% (Islamabad Local) plantlets proliferated buds, which initiated at the base of old leaves. In present study, the axillary buds grew quickly (except in Toro) and produced new buds. Within a short period of time, initial plantlets turned into a rich cluster of buds. Cultivar Chandler with small and thin buds proliferated at a high rate and produced light green coloration. Similar response of bud initiation was observed in Islamabad Local (with a reddish coloration at the base of culture). As compared to other cultivars, Toro produced buds with slow rate of proliferation, these buds were separable and not in a compact form as noted earlier. Cultivar Osogrande with a dark green colour and large sized buds, showed a high rate of proliferation than other cultivars. Similar results were obtained by Mahmood *et al.* (1994) who reported that the best shooting response was observed on the media supplemented with 0.5 mg/L BAP. Similarly, Ashrafuzzaman *et al.* (2013) reported that BARI Strawberry-1 produced highest average number of shoots (7) and the highest average length of shoot (3.34 cm) at the concentration of 0.5 mg/L BAP. However, Litwińczuk *et al.* (2009) applied gibberellic acid (0.1 mg dm^{-3}) in the media which stimulated the proliferation of axillary crown shoots and concurrently reduced the growth of callus as well as the formation of roots and the development of adventitious shoots.

Experiment 3.2. Clonal multiplication with MS media: Table 1 indicated that with increased concentration of BAP (0.5 to 1.5 mg/L), the rate of bud initiation also increased. Maximum number of buds (25 buds/culture) were proliferated by Osogrande when cultured on MS media containing 1.5 mg/L BAP and 0.1 mg/L IBA. With the same concentrations of BAP and IBA, Chandler and Islamabad Local initiated 20, and 15 buds/culture respectively. On the same media, Toro produced axillary buds with low rate of proliferation (5 axillary buds/culture). Marcotrigiano *et al.* (1984) observed that lowest level of BAP (0.3 mg/L) was as effective for shoots (or shoot buds) proliferation as 1.0 and 3.0 mg/L. Our results showed that 0.5-1.5 mg/L BAP played significant role in axillary buds proliferation. A change in the rate of buds initiation and proliferation was noted in Chandler and Osogrande, when axillary/adventitious buds were regularly subcultured on optimized media formulations. After 5-10 cycle of subculturing structural disorganization started in these cultivars. The rate of bud initiation was affected, either, the leaves became dominant over buds as in case of Chandler, or the buds produced short branched stems in Osogrande (with a decreased rate of bud initiation). However, in Toro and Islamabad Local the re-culturing cycle showed non-significant effect on bud initiation. Haddadi *et al.* (2010) reported that MS media with 2 µM TDZ and 4 µM BAP was optimum for shoot multiplication from the shoot tips. Similarly, Danso and Ford-Lloyed (2003)

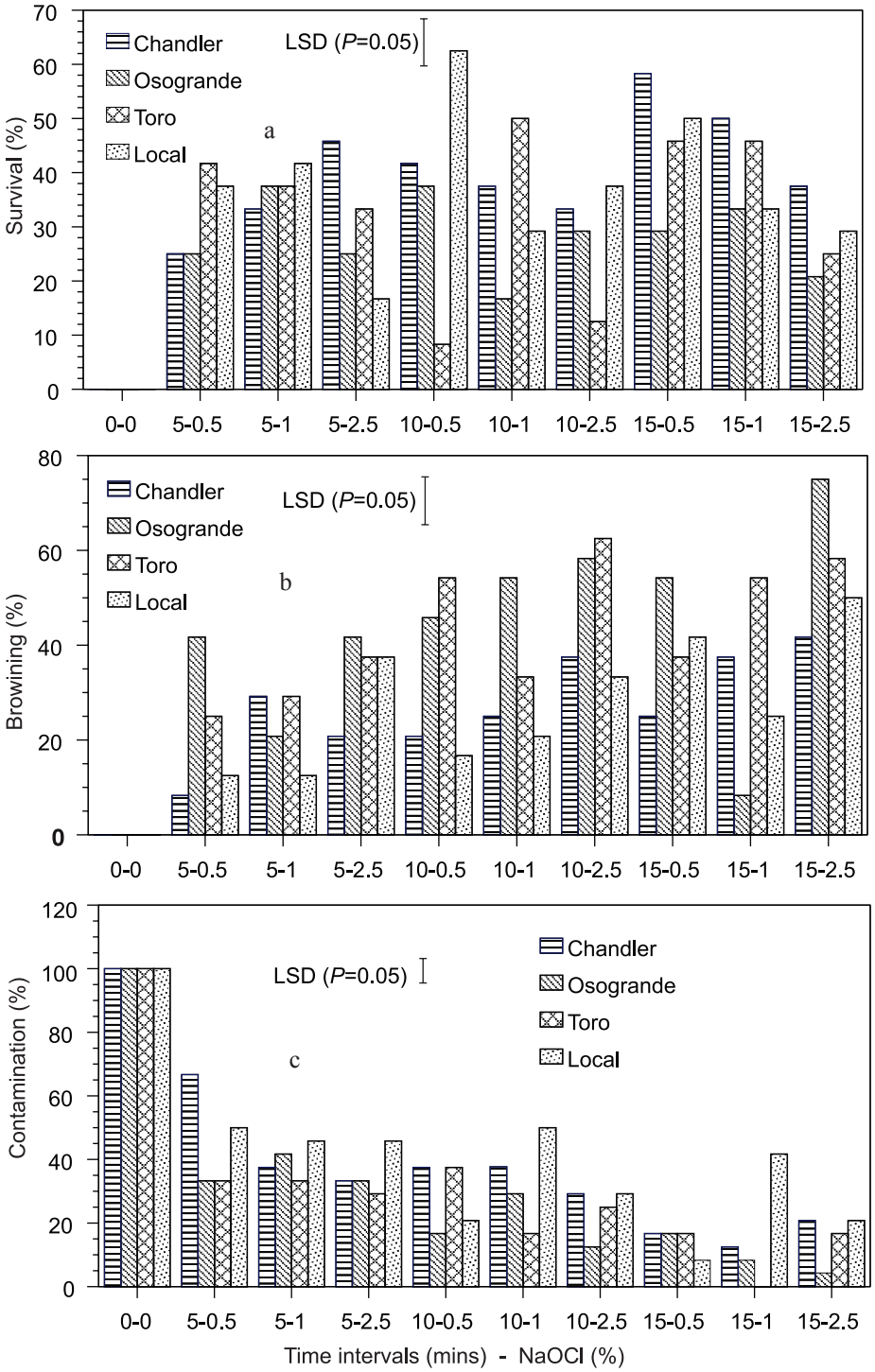

Fig. 3. Response of four strawberry cultivars *viz.,* Chandler, Osogrande, Toro and Islamabad Local to different treatments of NaOCl (0.5, 1 and 2.5%) at different time intervals (5, 10 and 15 minutes) on (a) survival, (b) browning and (c) contamination percentage of petiole segments explants. Vertical bars represent the least significant difference (LSD, *P*=0.05) within means.

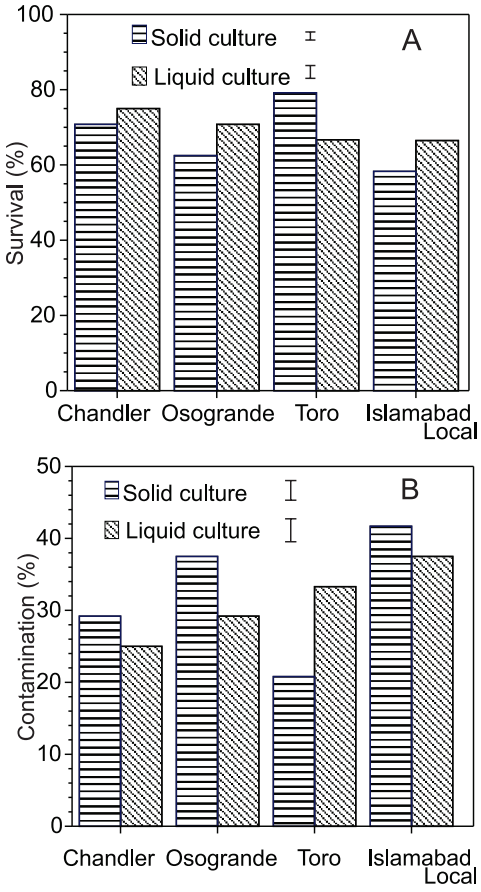

Fig. 4. *In vitro* response of meristem explants of four strawberry cultivars (Chandler, Osogrande, Toro and Islamabad Local) to solid and liquid media regarding (A) survival and (B) contamination percentage. Vertical bars represent the least significant difference (LSD, *P*=0.05) within means.

reported that the presence of BAP and NAA enhanced early development of shoot from encapsulated nodal cuttings of *Manihot esculenta*. Bhatt and Dhar (2000) obtained best explants establishment and shoot number per explant using MS media supplemented with 6-benzyladenine (4.0 µM) and α-naphthalene acetic acid (0.1 µM).

Clonal multiplication with Knop media: Maximum number of buds were initiated in Knop media containing 2 mg/L BAP concentration (Chandler, Osogrande, Toro), however, Islamabad Local showed varied response where 0.5 mg/L BAP concentration initiated maximum number of buds (Table 2). In all tested cultivars, Knop culture media produced reddish colour, specially in shoot segments, often without influencing growth rate. Boxus (1999) also recommended the use of Knop and MS culture media for clonal multiplication. Ahmad *et al.* (2002) reported that adventitious bud formation occurred *in vitro* in many strawberry cultivars during the proliferation phase on media containing Knop macronutrients, MS micronutrients, vitamins, aminoacids, 2.22 muM BAP, 2.46 muM IBA and 0.29 muM GA_3.

Experiment 3.3. Effect of sugar source on bud initiation: Data in Table 3 indicated that when a constant amount of glucose or sucrose (30 g/L) was used with different concentrations of kinetin, a high rate of bud proliferation (20 buds/culture) was noted in Osogrande on sucrose based media (0.8 mg/L Kin. and 0.2 mg/L NAA). However, when glucose was used with the same concentrations of kinetin and NAA, the proliferation rate decreased (5 buds/culture less than sucrose based media). On different concentrations of kinetin and NAA (Kin. 0 to 0.8 mg/L + NAA 0 and 0.2 mg/L), Chandler, Toro and Islamabad Local showed decline in the rate of bud initiation (2-3 bud/culture) when glucose based MS media was

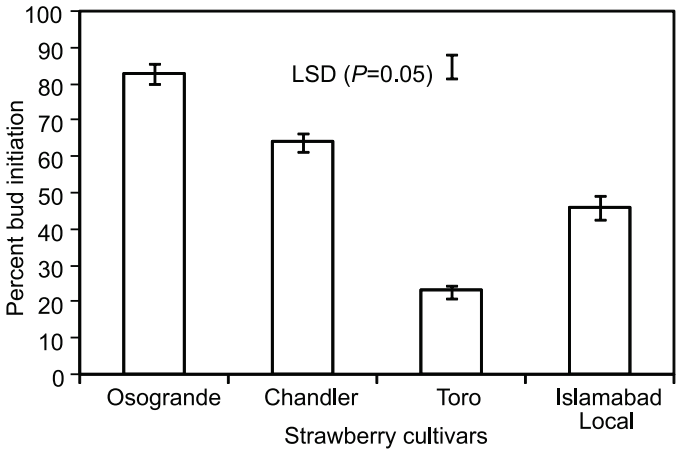

Fig. 5. Effect of BAP on *in vitro* produced plants through apical meristem. Vertical bars on data points represent the standard error within replicates whereas LSD (P=0.05) is the least significant difference within means.

Table 2. Knop Macro + MS Micro elements with growth regulators media for axillary/adventitious bud initiation

Growth regulators (mg/L)		Number of bud initiated			
BAP	IBA	Chandler	Osogrande	Toro	Islamabad Local
0.0	0.0	4 (±1.16)	3 (±0.58)	4 (±1.16)	4 (±1.16)
0.5	0.1	5 (±0.58)	10 (±1.16)	4 (±1.16)	15 (±1.16)
1.0	0.1	5 (±1.16)	15 (±1.16)	5 (±0.58)	10 (±1.16)
1.5	0.1	10 (±1.16)	15 (±1.16)	5 (±1.16)	5 (±0.58)
2.0	0.1	15 (±1.16)	15 (±0.58)	10 (±1.16)	10 (±0.58)
2.5	0.1	15 (±1.16)	10 (±1.16)	5 (±1.16)	5 (±0.58)
3.0	0.1	10 (±1.16)	10 (±0.58)	3 (±0.58)	4 (±1.16)

used. Sucrose is often assumed to be the best source of carbon for *in vitro* culture. More or less similar results were reported by Boxus (1999) when used glucose (0.12 M) in culture media, however, Miller (1986) suggested sucrose (0.058 M) containing media for bud initiation. Abdullah *et al.* (2013) reported that sucrose and glucose resulted in the greatest rate of proliferation in strawberry cultivar Elsanta, however, highest number of shoots were produced when MS media contained glucose (30 g/L). The sugars concentration lower or higher than 30 g/L reduced the number of shoots.

Generally, higher strawberry explants sterilization percentage can be obtained when they are disinfected with 0.5% NaOCl for 15 minutes. Solid MS medium containing 0.5 mg/L GA_3 can be used to enhance meristem explant survival rate. Similarly, MS medium containing 0.5 mg/L BAP significantly favoured bud initiation for cv. Osogrande. For clonal multiplication, maximum bud formation can be obtained in MS medium containing 1.5 mg/L BAP and 0.1 mg/L IBA. Likewise, the highest number of buds for cv. Osogrande can be achieved at sucrose based MS media containing 0.8 mg/L Kinetin and 0.2 mg/L NAA, however, it is 0.6 mg/L Kinetin and 0.2 mg/L NAA for cvs. Chandler, Toro and Islamabad Local.

Table 3. Effect of sugar source on bud initiation in MS media

Growth regulators (mg/L)		Number of bud initiated							
		Glucose				Sucrose			
Kinetin	NAA	C	O	T	IL	C	O	T	IL
0.0	0.0	4 (±1.16)	5 (±0.58)	3 (±1.16)	4 (±1.16)	3 (±0.58)	4 (±0.58)	3 (±0.58)	5 (±0.58)
0.2	0.2	5 (±0.58)	5 (±0.58)	3 (±0.58)	4 (±1.16)	5 (±0.58)	5 (±0.58)	4 (±1.16)	10 (±0.58)
0.4	0.2	5 (±0.58)	10 (±0.58)	5 (±0.58)	5 (±0.58)	10 (±1.16)	10 (±0.58)	5 (±0.58)	10 (±1.16)
0.6	0.2	10 (±1.16)	5 (±0.58)	5 (±1.16)	10 (±0.58)	15 (±0.58)	10 (±0.58)	5 (±0.58)	15 (±0.58)
0.8	0.2	10 (±1.16)	15 (±0.58)	5 (±0.58)	10 (±0.58)	15 (±1.16)	20 (±1.16)	3 (±0.58)	15 (±0.58)
$LSD_{0.05}$		1.53	0.73	0.78	1.24	1.24	1.1	1.1	1.16

Values in parenthesis are standard errors within replicates whereas as least significant differences and standard error differences among means are based on 5% level of probability. C = Chandler, O = Osogrande, T = Toro, IL = Islamabad Local

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Received: February, 2015; Revised: March, 2015; Accepted: March, 2015