

<http://www.pjbs.org>

PJBS

ISSN 1028-8880

**Pakistan
Journal of Biological Sciences**

ANSI*net*

Asian Network for Scientific Information
308 Lasani Town, Sargodha Road, Faisalabad - Pakistan

High Frequency, Direct Shoot Regeneration from Greenhouse-Derived Leaf Disks of Six Strawberry Cultivars

¹Fouad H. Mohamed, ²Mohamed S. Beltagi, ²Mona A. Ismail and ¹Ginesia F. Omar

¹Department of Horticulture, Faculty of Agriculture, Suez Canal University Ismailia, Egypt

²Department of Botany, Faculty of Science, Suez Canal University Ismailia, Egypt

Abstract: Leaf disks derived from either two-month-old GreenHouse-grown (GHD) strawberry plants or *in vitro* plantlets were cultured on MS media amended with 2 mg L⁻¹ Thidiazuron (TDZ), incubated for four weeks in the dark then for another four weeks under 16/8 h light regime. Regeneration capacity of leaf disks was compared to meristem-derived propagules in six strawberry cultivars. Direct shoot regeneration occurred in all tested cultivars with different frequencies depending on explant source. From GDH leaf disks, the cultivars Camarosa, Gaviota and Seascape produced the highest number of shoots/explant (38, 31 and 31 shoots, respectively). However, optimum number of shoots/explant from *in vitro* leaf disks was achieved in the cultivars Carlsbad, Chandler and Sweet Charlie (13.3, 12.6 and 12.3 shoots, respectively). In general, regeneration capacity of GHD leaf discs was more than two-folds of that obtained from *in vitro* leaf disks. The efficiency of meristem culture was intermediate between the above two systems. Rooted plantlets were successfully acclimatized under mist. The only morphological abnormality detected was a white streaked variant observed out of 456 Camarosa plants derived from meristem culture. SDS-PAGE of protein profile proved consistency in banding patterns of mother plants and those derived from direct regeneration or meristem proliferation.

Key words: Strawberry, direct shoot regeneration, SDS-PAGE protein

INTRODUCTION

The use of plant tissue culture technique has been proved as useful tool for mass propagation of several horticulture crops. The technique is important especially for clonal multiplication of virus-free stock plant materials in strawberry. For this purpose, runner meristem tip culture is normally utilized for true-to-type propagation of newly released cultivars and the method of propagation of strawberry was elaborated about thirty years ago (Boxus, 1999). Several reports indicated the possibility of *in vitro* regeneration of strawberry microplants via callus or cell suspension culture (Nehara *et al.*, 1990) or anther culture (Svensson and Johansson, 1994). However, this indirect regeneration pathway was often associated with genetic instability among regenerants, which alarmed nursery propagators (Quartia *et al.*, 1992; Palombi *et al.*, 2003). On the other hand, direct regeneration without intervening callus phase may provide useful alternative to the tedious, time consuming meristem culture. In this regard, direct shoot regeneration from strawberry leaf disks was described by several authors (Sutter *et al.*, 1997; Hassan, 1996), but the efficiency of the protocol was not compared to the standard meristem culture method.

Field performance of strawberry plants derived from meristem culture, callus culture and direct shoot regeneration from *in vitro* leaf disk was studied by Nehara *et al.* (1994). Variants with leaf chlorosis were detected in plants propagated by meristem or callus cultures, but not from those obtained through direct shoot regeneration. In other reports, Nehara *et al.* (1989) found increases in the percent of regenerated leaf disks and number of shoots per disk using leaves derived from one-month-old greenhouse plant materials, compared to older leaves. Only two, out of ten tested cultivars regenerated shoots. Therefore, propagation method must be taken into account when variety tests are performed and cultivar descriptions are made (Karthu *et al.*, 2001).

In this investigation, we report on the development of high efficiency direct shoot regeneration system from greenhouse-derived leaf disk explants of six North American strawberry cultivars. The current methods would be applicable in the micropropagation of genetically stable plant materials and identification of possible differences between plants regenerated directly from leaf tissues and those derived from meristem tip culture.

MATERIALS AND METHODS

The current study was conducted at The Plant Tissue Culture Lab, Department of Horticulture, Suez canal University, Ismailia, Egypt during the years 2004 and 2005. Six North American strawberry (*Fragaria* × *Ananassa* Duch.) cultivars: Carlsbad, Camarosa, Chandler, Gaviota, Seascape and Sweet Charlie were subjected to *in vitro* cultures from runner meristem tips, leaf disk regeneration from *in vitro* or greenhouse-derived leaf explants. In stage I, sterilized meristem tips (0.2-0.5 mm) were cultured on MS (Murashige and Skoog, 1962) medium supplemented with 30 g L⁻¹ sucrose, solidified with 7 g L⁻¹ agar and adjusted to pH 5.7 before being autoclaved at 121°C for 20 min. In stage II, meristem derived plantlets were multiplied for one month on the same medium amended with 0.3 mg L⁻¹ BA. In stage III, the obtained proliferated shoots were then subcultured into hormone-free MS medium for rooting and enlargement of single plantlets. For shoot regeneration, leaf disk explants (5×5 mm) taken from the previous plantlets were cultured with their adaxial surface facing the medium (MS+2 mg L⁻¹ TDZ). For each cultivars, twenty Leaf disks were tested for their regeneration capacity in small French square jars amended with 10 mL medium; one leaf disk was cultured per jar. Culture conditions were complete darkness for the first four weeks followed by four weeks of 16 h light/day provided by white fluorescent tubes (40 μmol S⁻¹ m⁻²) at 24±2°C. Parallel to the above, leaf disks (5×5 mm) from fully expanded leaves of two-month-old greenhouse-grown plants were excised, rinsed in tap water, sterilized in 10% Clorox solution for three min. followed by three washes in bidistilled sterile water and then cultured on regeneration medium (MS+2 mg L⁻¹ TDZ) following the same protocol and culture conditions reported above. The efficiency of *in vitro* proliferation via direct shoot regeneration was compared to meristem-derived regenerants.

Meristem tip cultures were carried out using runner tips from the three-month-old greenhouse-grown plants of the six tested cultivars. Runner tips were washed in running tap water, surface sterilized in 10% Clorox solution for 3 min, then washed three times in bidistilled sterile water. Meristem tips (0.5 mm) were isolated from runner tips and cultured directly on multiplication medium (MS+0.5 mg L⁻¹ BA) for proliferation under 16 h/day Light (40 μmol S⁻¹ m⁻²) at 24±2°C for six weeks. At least 20 meristem tips were utilized per each cultivar. Successfully regenerated shoot clumps from the three propagation systems were divided into single plantlets for further multiplication step in baby food jar (200 mL) contained

20 mL MS medium supplemented with 0.5 mg L⁻¹ BA for one month. The obtained cultures were then rooted in MS medium+1.0 mg L⁻¹ IBA. Culture conditions during the multiplication and rooting stages were similar to those reported for meristem cultures.

Rooting plantlets from each propagation system were transferred into the greenhouse for acclimatization under intermittent mist grown in small plastic pots amended with peatmoss and vermiculite (1:1 v v⁻¹). After three months of greenhouse growth, fully acclimatized plants were tested for possible morphological abnormalities. Measurements of regeneration capacity and the efficiency of each propagation method were performed after eight weeks from the initial leaf disk cultures in regeneration medium or six weeks from meristem tip cultures. Measurements included the number of regenerated shoots per leaf disk and percent regenerated shoots. The number of proliferated shoots from meristem and percent meristem developing shoots were also measured. Total numbers of rooted plantlets derived from one shoot clump obtained either by regeneration or proliferation were recorded upon taking plantlets for acclimatization.

Statistical analysis: All recorded data were analyzed using ANOVA at 5% significant level ($p = 0.05$) and mean separation by LSD (Steel and Torrie, 1980).

Histological analysis: From leaf disk regenerating shoot initials from four-weeks-old cultures, tissue samples were fixed in FAA solution, dehydrated in n-butyl alcohol, infiltrated and imbedded in pure paraffin wax. A rotary microtome was used to prepare serial thin sections (10 μ). The obtained sections were then stained with safranin and light green or hematoxylin.

Protein electrophoresis: Shoot samples from direct regeneration and meristem-derived plantlets were compared to runner propagated mother plants of the cultivar Gaviota based on banding patterns of SDS-PAGE protein. Total protein extracts were prepared by extracting appropriate weight from the frozen plant material with 0.125 M tris/borate, pH 8.9. All the obtained extracts were kept at 4°C for 24 h and then centrifuged at 10,000 rpm for 20 min. The supernatants were used for electrophoresis. SDS polyacrylamide gel electrophoresis (PAGE) was carried out with gel slabs according to the method of Laemmli (1970). Protein subunit bands were stained with comassie blue R-250 by standard technique. The gel was scanned using gel-pro-analyzer ver. 3.3 (Media Cybernetics, 93-97).

RESULTS

Direct shoot regeneration occurred from both *in vitro* leaf disk explants and greenhouse-derived leaf disks without callus formation. Regeneration was first visible after 4 weeks in greenhouse leaf disk and two weeks earlier than *in vitro* leaf explants. On both explant types, regeneration occurred from cut edges of the leaf explant as well as from abaxial surface, especially midveins (Fig. 1). Regeneration capacity in terms of average number of shoots per leaf disk were higher (29.5) in greenhouse-derived leaf disks than *in vitro*-derived ones (10.7) as an average of six cultivars (Table 1). However, the percentage of leaf disks forming shoots was higher in cultures initiated from *in vitro* leaf (90.2%) than greenhouse-derived leaf disks (78.4%).

Histological examination of regenerated leaf disks indicated the initiation of shoot primordium initials from the epidermal and sub-epidermal tissues. However, it was not clear whether the regenerant origin was either from a single or more than one cell (Fig. 1h). Compared to direct shoot regeneration from somatic leaf tissues, the proliferation rates of meristem tip explants were intermediate between those from greenhouse-derived and *in vitro* derived leaf disk explants. In all cultivars, strawberry runner meristem produced an average of 19.7 new shoots per explant on MS medium+0.5 mg L⁻¹ BA compared to 29.5 and 10.7 shoots/disk from greenhouse and *in vitro*-derived leaf explants grown on MS+2.0 mg L⁻¹ TDZ, respectively. Therefore, in term of the regeneration capacity of the three explants types, *in vitro* propagation methods could be ranked as: direct

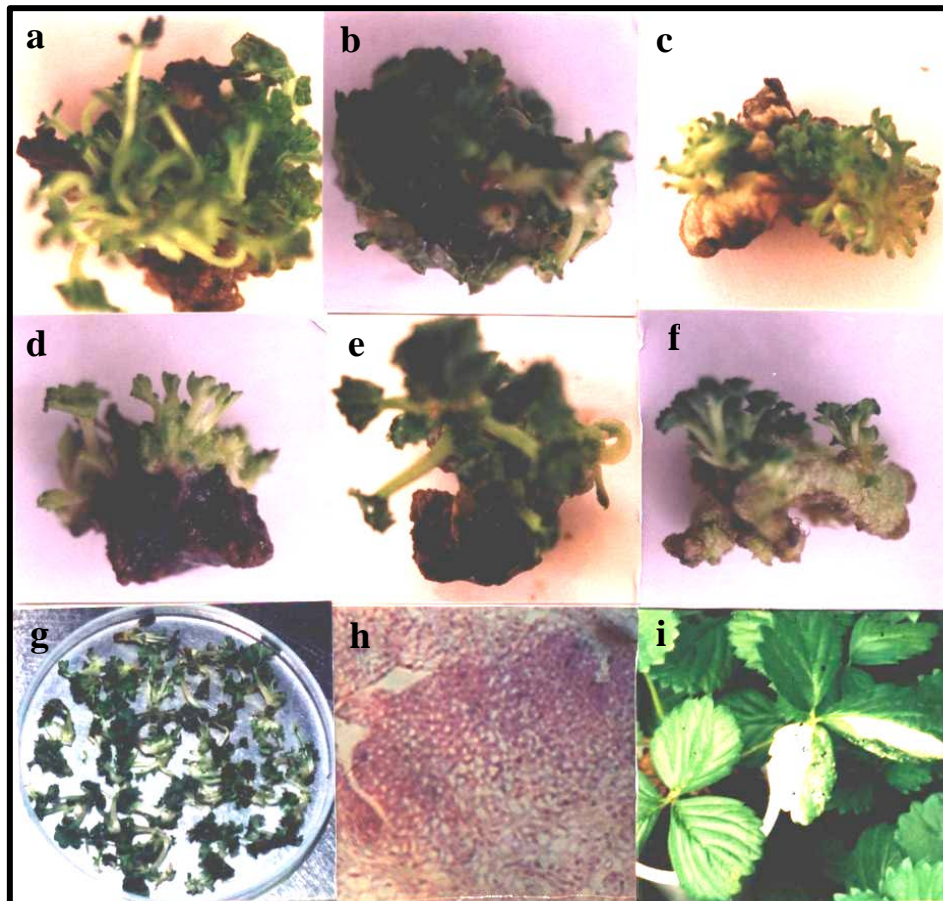


Fig. 1: Direct shoot regeneration (six weeks) for GHD leaf disks from strawberry cultivars: Camarosa (a), Gaviota (b), Seascape (c), Carlsbad (d), Chandler (e) and Sweet Charlie (f). Numerous plantlets after dividing one regenerated clump of "Gaviota" after 8 weeks (g), cross-section in regenerated leaf disk showing shoot meristem (h) and white streaked leaf derived from meristem culture (i)

Table 1: Percent explant forming shoots (%) and number of shoots regenerated per explant (No.) of six

Explant/ Cultivar	GHD leaf disk*		<i>In vitro</i> leaf disk*		Meristem tip**	
	(%)	No.	(%)	No.	(%)	No
Camarosa	88.3	38a	75.0	08.0c	80.0	18.0b
Chandler	69.4	25c	83.3	12.6ab	70.0	25.0a
Carlsbad	77.2	29bc	100	13.3a	75.0	24.6a
Gaviota	63.1	31b	100	03.3c	80.0	21.3ab
Seascape	78.0	31b	83.3	09.6b	80.0	16.0bc
Sweet charlie	93.7	23c	100	12.3ab	85.0	13.3d
Mean	78.4	29.5	90.2	10.7	78.3	19.7

* Regeneration on MS + 2.0 mg L⁻¹ TDZ, ** Proliferation on MS + 0.5 mg L⁻¹ BA

Table 2: Number of rooted plantlets obtained from three different explant sources after one subculture on multiplication media*

Culture	GHD leaf disk	<i>In vitro</i> leaf disk	Meristem tip
Camarosa	456a	115c	234b
Chandler	275d	138b	307a
Carlsbad	290c	133b	240b
Gaviota	403b	128bc	298a
Seascape	279d	95d	160d
Sweet charlie	434ab	172a	198c
Mean	356	239.5	130

*Rooting on MS+1.0 mg L⁻¹ IBA

regeneration from greenhouse-derived leaf disks> meristem-derived explants>*in vitro*-derived leaf disk explants. The same ranking was also true for the percentage of explants formed shoots.

Genotypic variability in shoot forming capacity was also detected, (Table 1 and Fig. 1a-f). The highest number of shoots regenerated per green house-derived leaf disk was found in Camarosa (Table 1 and Fig. 1g) with an average of 38 shoots/leaf disks, followed by Gaviota, Seascape, Carlsbad, Chandler and Sweet Charlie. However, using leaf disk from *in vitro* grown plantlets, the regeneration capacity was in the order: Carlsbad > Sweet Charlie ≥ Chandler > Seascape > Gaviota ≥ Camarosa. Chandler followed by Carlsbad and Gaviota produced more shoots per proliferated meristem tip than Camarosa, Seascape and Sweet Charlie. However, Sweet Charlie propagated by direct shoot regeneration from greenhouse-derived leaf disk had the highest frequency of shoot regeneration (93.7%) followed by Camarosa (88.8%). The percentages of regenerated shoots from the *in vitro*-derived leaf disks were highest (100%) in Sweet Charlie and Carlsbad, followed by 83% in Chandler and Gaviota, but 75% in Camarosa. Regeneration of shoot from meristem tips occurred in 85% of the cultured explants of Sweet Charlie and in 80% of Camarosa, Gaviota and Seascape and was the lowest (70%) in Chandler (Table 1). Upon transferring regenerated clumps into rooting medium, the highest number of rooted shoots obtained after one month were in those derived from regenerated clump of greenhouse-derived leaf disks and

Table 3: Comparative analysis of relative concentration, molecular weight (Mol. Wt.) and mobility rate (R_m) of proteins of cultured strawberry derived from three different explants. Bands were separated using SDS-PAGE technique

Band No.	Band % and explant type			R _m	Mol. Wt (KDa)
	I	II	III		
1	4.68	0.00	5.16	0.06	256.88
2	0.40	0.53	1.05	0.11	199.30
3	0.96	0.58	0.92	0.12	182.40
4	0.84	0.29	0.00	0.16	144.55
5	0.00	0.41	0.00	0.19	127.95
6	2.46	4.31	2.47	0.22	111.32
7	0.00	0.00	0.72	0.25	102.87
8	3.03	3.03	1.29	0.27	099.00
9	2.96	2.30	1.33	0.33	082.51
10	2.29	2.07	1.49	0.35	076.86
11	2.22	1.71	1.17	0.37	073.94
12	4.60	3.34	2.79	0.43	061.45
13	9.20	7.65	9.30	0.45	058.11
14	4.67	4.76	3.55	0.52	049.24
15	2.88	3.25	3.61	0.55	047.00
16	1.85	1.87	1.12	0.58	044.93
17	4.14	4.32	5.10	0.64	040.80
18	2.21	2.06	3.12	0.71	036.88
19	9.70	8.28	11.4	0.19	032.11
20	0.92	1.29	1.36	0.83	029.87
21	0.73	0.52	2.71	0.87	026.14
22	0.28	0.30	1.19	0.90	023.91
23	7.79	6.75	6.98	0.97	018.94
24	6.41	4.00	4.84	0.99	017.35
Total bands	22	22	22		

I, II and III = Mother plant, GHD regenerant and meristem derived plant

subcultured onto multiplication medium before rooting with an average of 356 rooted plantlet (mean of six cultivars). In comparison, shoot clump from meristem explants and *in vitro* derived leaf disk produced an average of 239.5 and 120 rooted plantlets, respectively, indicating the high efficiency of direct shoot regeneration from greenhouse isolated leaf disks for micropropagation.

Strawberry cultivars also differed in the number of rooted shoots obtained in the final rooting stage (Table 2). More than 400 rooted plantlets were obtained from the cultivars Camarosa, Sweet Charlie and Gaviota. On the other hand, Chandler and Gaviota produced the largest number of rooted plantlets propagated from meristem tips; while, Sweet Charlie produced the highest number of rooted plantlets from proliferated *in vitro* leaf disk explant.

In vitro propagated plantlets were successfully acclimatized under mist in the greenhouse and formed normal root and shoot systems suitable for further propagation in nursery field. No morphological drift in leaf shape or serration pattern were observed except one plant with sectorial leaf chlorosis (Chimera) detected from 456 meristem-derived Camarosa plantlets (Fig. 1i).

The SDS-PAGE protein patterns (Fig. 2 and Table 3) revealed relative similarities concerning the total number of protein bands (22 bands) appeared in each of the three

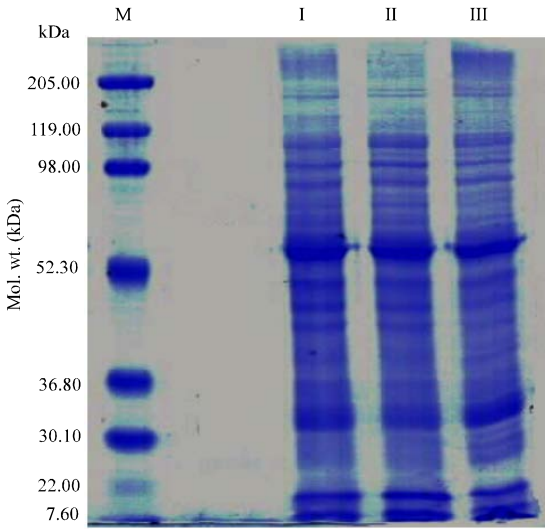


Fig. 2: Electrophotograph of Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis (SDS-PAGE) of proteins of cultured strawberry derived from there different explants: mother plant, GHD-regenerant and meristem-derived plants; M, reference protein molecular weights

tissue samples derived from mother plants, regenerates from two-month-old greenhouse leaf disk or meristem-derived plants (Table 3). Moreover, 20 out of 22 bands (91% consistency) appeared in the three protein profiles. On the other hand, the amount of protein in each band (band %) was not always the same at each rate of mobility (R_m). Qualitatively, few (one or two) protein bands appeared and/or disappeared relative to source of tissue tested.

DISCUSSION

The present study indicated the possibility of obtaining an efficient, high frequency direct regeneration of shoots from leaf disks isolated from two-month-old greenhouse-grown strawberry plants on MS medium + 2 mg L⁻¹ TDZ. In this system, the number of regenerated shoots was the highest among all previously reported *in vitro* strawberry regeneration systems. Nehara *et al.* (1990) used complex additions of auxins and cytokinines to obtain *in vitro* shoot regeneration in different strawberry cultivars. In most cases, regeneration occurred indirectly via callus which might not be suitable protocol if genetic stability is required. Sorvari *et al.* (1993) and Hassan (1996) obtained direct shoot regeneration from *in vitro* leaf disks. However, their regeneration capacities were far less than those obtained from greenhouse-

derived leaf disk explants, but similar to those obtained from *in vitro* leaf disk in the current study, indicating the importance of the explant type in any regeneration protocol (Nehara *et al.*, 1989). *In vitro* derived leaves are less photosynthetically active and may contain less endogenous hormonal balance as compared to older leaves developed from larger crowns in the greenhouse-grown plants. In addition, we used to observe the disappearance of *in vitro* developed leaves during their *ex vitro* acclimatization period and the emergence of new, greener and actively growing leaves from the enlarged crown. Subsequently, segments from the later leaves may result in increased regeneration capacity on a regeneration medium.

Explants from runner tips regenerated more shoots than those obtained from *in vitro* leaf explant, but less than those from greenhouse-derived ones. Aside from their *in vitro* development in BA instead of TDZ-amended medium, their tissues are older than *in vitro* and younger than *ex vitro* leaf disk explants, perhaps leading to intermediate regeneration capacity.

The high regeneration capacity we obtained with TDZ as the sole growth regulator in the medium may be attributed to its extreme high cytokinin activity (Mok *et al.*, 1982) and its stability in plant tissue (Mok and Mok, 1985). The efficiency of TDZ for *in vitro* regeneration of strawberry was also reported by Svensson and Johansson (1994), where the frequency of regeneration was double when BA was replaced by TDZ with the same concentration (5 μ M). In other reports, very high level of TDZ (60-80 μ M) was required for 100% regeneration from strawberry leaf disks (Sutter *et al.*, 1997).

Holding fairly to our observations, the appearance of white streaked leaf variants was previously reported in micropropagated strawberry by Lis and Masny (2000). These variations were only confined to plants propagated by meristem or callus but not from direct regeneration systems (Nehara *et al.*, 1994) and were mostly related to explant origin (Popescu *et al.*, 1997). Cultivar-dependent variation in shoot regeneration capacity was previously reported in our lab in several crops including cucumber (Ismail, 2000), tomato (Ismail, 2003) and strawberry (Hassan, 1996; Mohamed, 2002).

The results of SDS-PAGE indicated stability in protein patterns of either *in vitro* regenerants from greenhouse-derived leaf disks or meristem tip explants which had the same anabolic capacity to runner-propagated mother plants for gene-encoded polypeptides. Brandizzi *et al.* (2001) found that DNA variation induced by *in vitro* culture of strawberry could be lost after transferring the plants to the greenhouse, indicating their

epigenetic nature. These, along with the observed phenotypic stability of plants derived from direct shoot regeneration, indicate possible uses of these regenerants for efficient micropropagation of strawberry from virus-indexed nuclear stocks.

REFERENCES

- Boxus, P., 1999. Micropropagation of Strawberry via Axillary Shoot Proliferation. In: Plant Cell Culture Protocols. Methods in Molecular Biology. Part III. Plant Propagation *In vitro*. Hall, R.D. (Ed.) Humana Press Inc., Totowa NJ, 111: 103-114.
- Brandizzi, F., C. Forni, A. Fattarelli and C. Damiano, 2001. Comparative analysis of DNA nuclear content by flow cytometry on strawberry plants propagated via runners and regenerated from meristem and callus cultures. *Plant Biosystemms*, 135: 169-174.
- Hassan, M.A., 1996. *In vitro* shoot regeneration from strawberry leaf tissues. *Zagazig J. Agric. Res.*, 23: 101-113.
- Ismail, M.A., 2000. Studies on the effects of genotypic variability, source of explant and culture medium on callus growth and organogenic capacity of *Cucumis sativus* plants. M.Sc. Thesis, Fac. Sci., Suez Canal Univ., Egypt.
- Ismail, M.A., 2003. Genotypic responses of cultured tomato plants to salt stress. Ph.D Thesis, Fac. Sci., Suez Canal Univ., Egypt.
- Karhu, S., S. Sorvari, E. Kanervo and S. Pihakaski, 2001. Growth characteristics of micropropagated strawberries. *Acta Hort.*, 560: 539-542.
- Laemmli, U.K., 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature*, 227: 680-685.
- Lis, E. and A. Masny, 2000. Phenotypic instability of micropropagated Real Strawberry plants. *Zeszyty-Naukowe-Inst. Sadownictwa-i-Kwiaciarnstwa-w-Skierniewcach*, 8: 269-273.
- Mohamed, F.H., 2002. Responses of six strawberry cultivars to salinity during the *in vitro* proliferation and rooting stages and post-acclimatization period in the greenhouse. *Zagazig J. Agric. Res.*, 29: 767-792.
- Mok, M.C., D.W. Mok, D.J. Armstrong, K. Shudo, V. Isogal and T. Okamoto, 1982. Cytokinin activity of N-phenyl-N-thiadiazol-5 ylurea (Thidiazuron). *Phytochemistry*, 21: 1509-1511.
- Mok, M.C and D.W. Mok, 1985. The metabolism of [¹⁴C] thidiazuron in callus tissues of *Phaseolus lanatus*. *Physiol. Plant.*, 65: 427-432.
- Murashige, T. and F. Skoog, 1962. A revised medium for rapid growth and bioassays with tobacco tissue cultures. *Physiol. Plant.*, 15: 473-497.
- Nehara, N.S., C. Stushnoff and K.K. Kartha, 1989. Direct shoot regeneration from strawberry leaf disks. *J. Am. Soc. Hortic. Sci.*, 114: 1014-1018.
- Nehara, N.S., C. Stushnoff and K.K. Kartha, 1990. Regeneration of plants from immature leaf derived callus of strawberry (*Fragaria* × *Ananassa*). *Plant Sci.*, 66: 119-126.
- Nehara, N.S., K.K. Kartha, C. Stushnoff and K.L. Giles, 1994. Effect of *in vitro* propagation methods on field performance of two strawberry plant cultivars, *Euphytica*, 76: 107-115.
- Palombi, M.A., S. Monticelli, A. Festa, C. Damiano, A.S. Economon and P.E. Reed, 2003. *In vitro* adventitious regeneration affects the genetic stability of strawberry plants (*Fragaria* × *Ananassa* Duch.) *Acta Hort.*, 616: 459-462.
- Popescu, A.N., V.S. Isa, M.S. Coman, M.S. Radulescu, H.V. Scheer, F. Lieten and J. Dijkstra, 1997. Somaclonal variation in plants regenerated by organogenesis from callus cultures of strawberry (*Fragaria* × *Ananassa*). *Acta Hort.*, 439: 89-96.
- Quartia, R., D. Nati and F.M. Paoloni, 1992. Strawberry anther culture. *Acta Hort.*, 300: 335-339.
- Sorvari, S., S. Ulvinen, T. Hietaranta and H. Hiirsalmi, 1993. Preculture medium promotes direct shoot regeneration from micropropagated strawberry leaf disks. *HortScience*, 28: 55-57.
- Steel, R.G.D. and G.J. Torrie, 1980. Principles and procedures of Statistics. A Biometrical Approach. 2nd Edn. McGraw Inc., USA.
- Sutter E.G., H. Ahmadi, J.M. Labavitch, A. Altman and M. Ziv, 1997. Direct regeneration of strawberry (*Fragaria* × *Ananassa* Duch.) from leaf disks. *Acta Hort.*, 447: 243-245.
- Svensson, M. and L.B. Johansson, 1994. Anther culture of *Fragaria* × *Ananassa*: Environmental factors and medium components affecting microspore divisions and callus production. *J. Hortic. Sci.*, 69: 417-426.