Romanian Biotechnological Letters *Vol. 15, No.2, Supplement, 2010*

ORIGINAL PAPER

In vitro culture medium and explant type effect on callogenesis and shoot regeneration in two genotypes of ornamental strawberry

Received for publication, December 20, 2009
Accepted, February 5, 2010

ANCA NICOLETA SUTAN[*], AUREL POPESCU[*], VALENTINA ISAC[**]
[*] *University of Pitesti, Faculty of Sciences, Department of Biology and Horticulture, Pitesti, Romania*
[**] *Research Institute for Fruit Growing, Pitesti, Romania*
Corresponding author: Anca Nicoleta Sutan, University of Pitesti, Faculty of Sciences, Department of Biology and Horticulture, Targul din Vale, no. 1, 110040 Pitesti, Romania, tel.: 0040 348 453100, fax: 0040 348 453123, e-mail: ancasutan@yahoo.com

Abstract

As some of the ornamental varieties of strawberry obtained from Fragaria x Potentilla crosses are lacking the ability to form runners, their in vitro propagation is dependent on either direct or indirect organogenesis. The influence of culture medium composition and explant type were investigated in two genotypes of ornamental strawberry, "Pink Panda" and "Serenata", respectively, in order to establish an efficient protocol for regeneration by indirect organogenesis. Aiming to a good rate of callogenesis and shoot regeneration, the effect of different combinations and concentration of growth regulators (2,4-D, IBA, and BAP) added in culture media (either MS or LF) were evaluated with leaf and petiole explants. It was found that the highest frequency of explants forming callus have been induced in both varieties investigated on the LF basal medium containing 0.5 mg/l or 1.0 mg/l 2.4-D and, respectively, 3.0 mg/l BAP. A maximum of 100% leaf explants, and 92% petiole explants formed calli having characteristics of those regenerating shoots in "Serenata" variety. Similarly, a maximum of 92% petiole explants formed callus in "Pink Panda" intergeneric variety.

Key words: callus induction, genotype, explant type, culture medium, plant growth regulators.

Introduction

Sexual compatibility of *Potentilla palustris* with some *Fragaria* species (Niemirowicz-Szczytt, [13]; Sayegh and Hennerty, [17]), allowed the occurence of a large range of *Fragaria x Potentilla* intergeneric hybrids, combining the ornamental value given by the beauty of their flowers and prolonged blossoming season (May - October) with production of edible fruits. Because the genetic limitations associated with high heterozygosity and polyploidy which hamper the traditional breeding methods, the clonal propagation of intergeneric hybrids *Fragaria x Potentilla* provides an advantage for the multiplication of these elite plants without sexual recombination.

Callogenesis and regeneration of callus culture via shoot organogenesis seem to be highly dependent of a number of factors, such as ratio of auxins and cytokinins in the culture medium (Dewitte and Murray [4]), the genotype (Landi and Mezzetti [7]), and the source of the explants used. Different cultivars of strawberry have shown large variability in the differentiation competence of their somatic tissue, and many workers have reported the use of different combinations of growth regulators for shoot regeneration from different types of explants (Schaart *et al*. [18]; Passey *et al*. [14]; Zhao *et al*. [23] ; Yonghua *et al*. [21]; Biswas [2]).

The objective of this study was to find the most appropriate type of explant and to investigate the specific nutrient requirements for obtaining callus tissue from leaf and petiole explants in "Pink Panda" and "Serenata" intergeneric hybrids derived from *Fragaria x*

Potentilla crosses, in order to establish an highly efficient protocol for large scale propagation of new varieties of ornamental strawberry developed through breeding and selection.

Materials and methods

Plant material. "Pink Panda" and "Serenata" genotypes from the *Fragaria* Germplasm Collection of the Institute for Fruit Growing, Pitesti, Maracineni, Romania were cultured *in vitro* starting from either meristems or self-pollination derived seeds, on LF basal medium. Leaf disks and petiole segments collected from six weeks old *in vitro* plantlets, precultured for two weeks on LF basal medium containing 0.5 mg/l BAP, 0.5 mg/l IBA and 0.2 mg/l GA_3 (Sorvari *et al*. [19]), were used as explants.

Callus induction experiments. Callus induction media consisted of Murashige and Skoog [11] and Lee and Fossard [8] basal media, each of them supplemented with various combinations of plant growth regulators, a total of six different combination for each variety and explant type, respectively, being tested (Table 1). Dextrose, at a concentration of 40 g/l, was used as carbon source in all culture media.

Table 1. Combination and concentration of plant growth regulators added in basal media in order to induce callus formation.

Callus induction media code	Basal culture media	Combination and concentration of plant growth regulators (µM)		
		2,4 - D	IBA	BAP
CIM 1	MS, LF	2,7	-	1.3
CIM 2	MS, LF	4.5	-	1.3
CIM 3	MS, LF	4.5	-	2.2
CIM 4	MS, LF	-	2.5	1.3
CIM 5	MS, LF	-	4.9	1.3
CIM 6	MS, LF	-	4.9	2.2

Both the leaf explants (0.3-0.5 mm diameter) and petiole segments (0.3-0.5 mm) were randomly placed onto the culture medium. The leaf explants were placed with their abaxial surface in contact with the medium. After two weeks of incubation in the dark, in the growth chamber at the temperature of 22-24°C, the cultures were maintained under a photoperiod of 16 hours light/8 hours darkness and a light intensity of about 40 µmol m^{-2} s^{-1}. The number of explants forming calli was scored after 45 days of culture for both varieties.

Shoot organogenesis. Callus cultures initiated from the leaf and petiole-derived calli were maintained on the same medium whithout subculturing them, until the shoots induction. The number of shoots formed per callus was determined after five months in culture under a 16 hour light photoperiod.

Experimental design and statistical analysis. The rate of callogenesis (%) was determined as the ratio of the number of explants that developed calli to the total number of explants. To avoid major statistical errors, all of the experimental treatment (two genotypes x two type of explants x two type of basal media x six growth regulators combinations) was performed with five replicates (in conical flasks with 30 ml of culture medium, closed with cotton-wool bungs and tinfoil) of six somatic explants. Analysis of variance was used to analyse the effects and interactions of the four main factors (genotype, basal medium, combination and concentration of PGR and explant types) on induction of callogenesis. Statistical analysis of the data obtained with "Pink Panda" and "Serenata" varieties respectively, on basal media containing different concentrations of auxins for callus induction, were performed using Duncan's Multiple Range test, at $p < 0.05$ and Independent Sample T-

Test, working with Statistical Package for the Social Science (SPSS) statistical software (version. 16.0).

Results

The callogenic response was induced after 3 weeks of *in vitro* culture, in response to IBA or 2.4-D and BAP added in MS and LF basic media. These calli were formed all over the surface of the leaf explants and were predominantly initiated at the cut edges of the petiole segments, directly in contact with the culture medium. It is known that plant hormones are present in higher quantities after wounding and are involved in cell proliferation at the wound site (Khal [6]). Callus cultures exhibited a pink pigmentation of anthocyanins, in both leaf and petiole explants of "Serenata" variety.

Effect of genotype. Significant differences on callus induction and production was observed between the two genotypes of ornamental strawberry. Irrespective of the basic culture medium, plant growth regulators combination and concentration, or explant type, the rate of callogenesis was found to be, generally, higher in "Serenata" than in "Pink Panda" variety. Differences was less pronounced when petiole explants were cultured on MS basic medium or indicated a better performance of "Pink Panda" variety on LF medium supplemented with IBA and BAP (Fig.1).

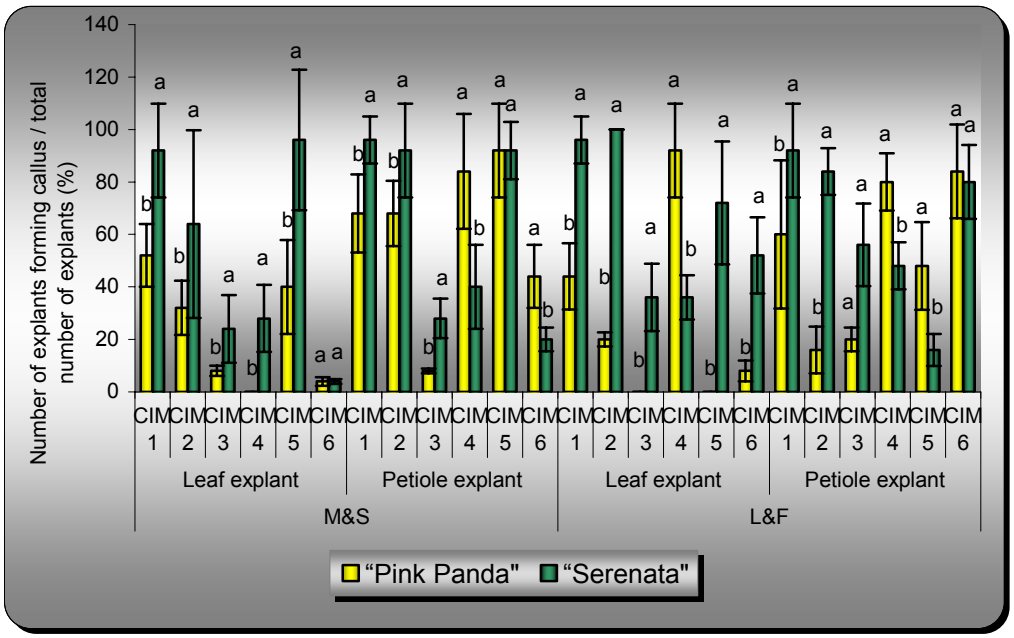


Fig. 1. The influence of genotype on the callogenesis ability of leaf and petiole explants
(bars represent standard deviation; letters above the columns indicate percentages significantly different from each other, $p<0.05$).

Effect of the culture medium composition. Considering both types of somatic explants, culture medium composition significantly influence callus induction in ornamental strawberry genotypes investigated. The highest rates of callus induction was 100% from leaf explants and 92% from petiole explants (Fig. 3), respectively, in LF basal medium, containing 2.7 µM or 4.5 µM 2.4-D and 1.3 µM BAP (Table 1), indicating a more adequate composition of nutrients to the *in vitro* regeneration requirements of this intergeneric hybrid. Thus, in "Pink Panda" variety, the rate of callogenesis was 44% for leaf explants and 60% for petiole explants, respectively, in media with the same concentration of 2.4-D and BAP. In "Serenata" variety, the ability of leaf and petiole explants to produce callus was higher on culture media

containing low concentrations of 2,4-D and BAP. As shown in Figure 1, an inhibition of callogenesis occurred for both types of explants on media with an increased BAP concentration (2.2 µM, instead of 1.3 µM).

The plant growth regulators had significant effects on the induction of callus formation in "Pink Panda" variety. The highest overall percentage of leaf explants forming callus (92%) was induced in LF basal medium with low concentrations of IBA (2.5 µM) and BAP (1.3 µM). Surprisingly, the leaf explants failed to form callus on MS basal medium supplemented with the above mentioned combination of growth regulators. However, the same combination of growth regulators promoted callus formation from the petiole explants on both LF and MS media, with a frequency of 84% and 80%, respectively.

When added to the MS basal medium, the combination of 2.2 µM BAP and 4.9 µM IBA (Fig. 2) promoted callus proliferation in both leaf and petiole explants from "Serenata" variety, while only petiole explants were induced to form callus in "Pink Panda" variety.

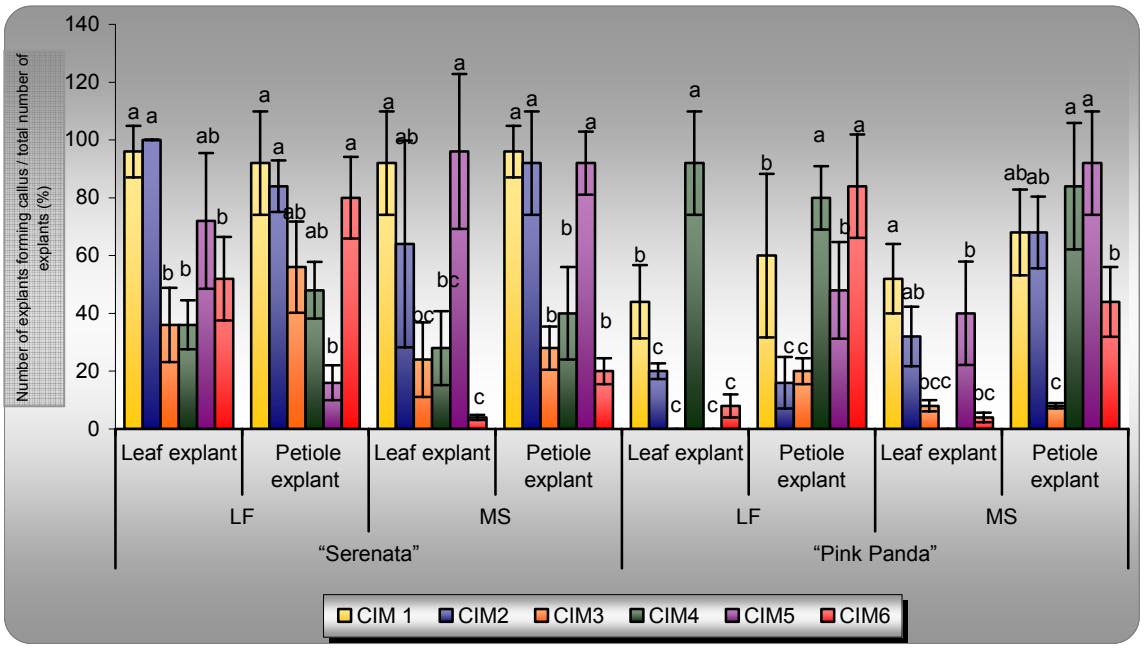


Figure 2. The effect of culture medium on the callogenesis ability of leaf and petiole explants
(bars represent standard deviation; letters above the columns indicate percentages significantly different from each other, $p<0.05$).

Effect of explant types. Statistical analysis revealed significant differences in callus induction frequency not only between the two genotypes, but also between somatic tissues used as explants. "Serenata" variety showed the highest percent of leaf explants (100%) forming callus on LF basal medium. Petiole explants proved to be the explants type with the highest percentage of callus formation when cultured on MS basal medium, for both varieties of ornamental strawberry investigated (Fig. 3).

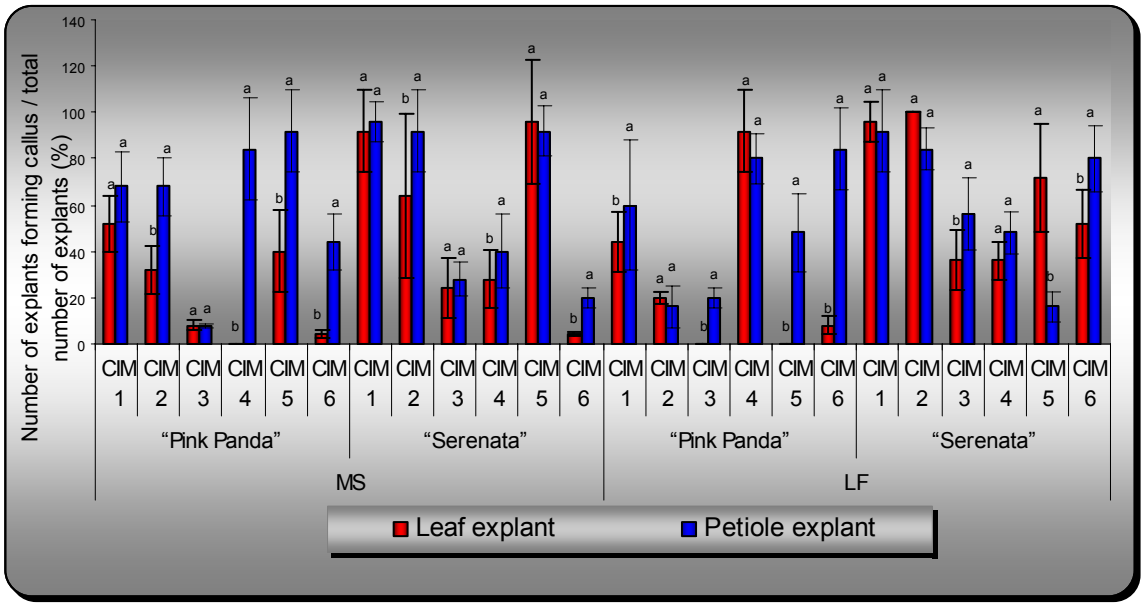
Shoot organogenesis. Comparing with callus induction and proliferation, regenerative ability of tissue-derived calli was relatively low in "Serenata" variety. Moreover, calli formed from explants of "Pink Panda" variety were unable to regenerate shoots on media used in this study. Shoots regeneration was obtained in "Serenata" variety only from those explants which previously showed a high frequency of callus formation (Fig 1). The frequency of shoot regeneration from leaf-derived calli cultured on LF basal medium supplemented with 2.5 µM IBA and 1.3 µM BAP (36%) was significantly higher compared to those cultured on MS basal medium supplemented with 4.9 µM IBA and 1.3 µM BAP (22%). Shoot regeneration was induced also, but at a low frequency (10%), when petiole explants were cultured on LF

basal medium, supplemented with 2.5 µM IBA and 1.3 µM BAP. For this genotype, the callus induction frequency and plant regeneration ability appeared to be in an inverse relationship.

Table 2. Percentages of leaf and petiole explants showing shoot formation in "Serenata" variety of intergeneric hybrid *Fragaria* x *Potentilla*

	Basal culture medium	PGR combinations	Explant type forming shoots (%)	
			Leaf explants	Petiole explants
Cv. "Serenata"	LF	2.5 µM IBA + 1.3 µM BAP	36 ± 2.3 a	10 ± 1.1 a
	MS	4.9 µM IBA + 1.3 µM BAP	22 ± 1.6 b	-

Data are expressed as means ± SE. Means in the same column that are followed by different letters are significantly different ($p<0.05$) using Duncan's Multiple Range Test.



(bars represent standard deviation; letters above the columns indicate percentages significantly different from each other, $p<0.05$).

Fig. 3. The ability to form callus of leaf and petiole explants as influenced by the interaction between genotype and culture medium composition.

Discussion

Plant genotype, explant types, basal culture medium and PGR combinations and concentrations, significantly influenced the frequency of callus formation and shoot regeneration, respectively, in "Serenata" and "Pink Panda" varieties.

In terms of the number of explants which formed callus, the petiole explants gave a better response than the leaf explants. Many other authors reported that both callus induction and shoot organogenesis were dependent on the explant source (Passey [14]; Debnath [3]). However, our results proved that is no correlation between callus formation ability and shoot formation capacity, as in "Serenata" variety, whose leaf explants exhibited a low ability to form callus, but leaf-derived calli regenerated shoots at a relatively high percentage. The better regenerative ability of leaf explants can be attributed to the size of explants. In this respect, Pierik [16] reported that larger explants sometimes regenerate easier that smaller ones, and that the larger explants produce more shoots *in vitro*.

This study showed that basal culture medium and plant growth regulators types, concentrations and combinations are another key factors regulating callus induction and shoot

organogenesis. Callus induction frequency was affected by concentration of BAP and the effect of auxins on callus induction was genotype – specific. In low concentration 2,4-D has been shown to be the most effective for callus induction in "Serenata" variety, irrespective of explant type cultured. As have been reported, 2,4-D induce callus formation in a variety of species (Ma and Xu [9], Thao *et al*. [20]). In contrast, IBA added in low concentrations in culture medium, was most effective in stimulating callus formation, in "Pink Panda" variety. These observations were similar to those noted by other autors (Nehra *et al*. [12] ; Barcelo *et al*. [1] 1998; Gruchala *et al*. [5])

Presumably, maintainance of tissue-derived calli in culture without their transfer to the fresh medium may be the reason for the low regeneration frequency in "Pink Panda" and "Serenata" varieties, respectively, or even the lack of regeneration in the case of leaf-derived calli. On the other hand, while IBA in combination with BAP stimulated shoot organogenesis in "Serenata" intergeneric hybrid of *Fragaria x Potentilla*, 2,4-D has proven inappropriate for shoots regeneration in both investigated genotypes of ornamental strawberry. Moreover, anthocyanin production does not influence callus induction in "Serenata" variety, according with Mori *et al*. [10].

Genotype was proven to be a critical factor for indirect shoot organogenesis in intergeneric hybrids *Fragaria* x *Potentilla*. There were distinct differences in callus formation ability and shoots regeneration frequency between the two ornamental strawberry varieties investigated. Significant variations in callus formation and shoot regeneration ability of the different genotypes have been previously reported in strawberry (Barcelo *et al*. [1]; Passey *et al*. [14]; Landi and Mezzetti [7]). Considering that specific genes are involved in shoot organogenesis (dedifferentiation, acquisition of competence and induction), as reported by Phillips [15], it is likely that, in some genotypes, genes involved in shoot organogenesis may be suppressed due to inappropriate culture condition.

REFERENCES

1. M. Barcelo, I. El-Mansouri, J.A. Mercado, M. Quesada, F.P. Alfaro, Regeneration and transformation via *Agrobacterium tumefaciens* of the strawberry cultivar Chandler. Plant Cell Tiss. Org. Cult., 54: 29-36 (1998).
2. M.K. Biswas, U.K. Roy, R. Islam, M. Hossain, Callus culture from leaf blade, nodal, and runner segments of three strawberry (*Fragaria* sp.) clones. Turk. J. Biol., 33: 1-6 (2009).
3. C.S. Debnath, Strawberry sepal: Another explant for thidiazuron-induced adventitious shoot regeneration. *In Vitro* Cell. Dev. Biol. Plant, 41: 671-676 (2005).
4. W. Dewitte, J.A. Murray, The plant cell cycle. Anuu. Rev. Plant. Biol., 54: 235-264 (2003).
5. A. Gruchala, M. Korbin, E. Żurawicz, Conditions of transformation and regeneration of "Induka" and "Elista" strawberry plants. Plant Cell Tiss. Org. Cult., 79: 153-160 (2004).
6. G. Kahl, Wound repair and tumor induction in higher plants. In: T. Akazawa & H. Imasei (eds). The New Frontiers in Plants Biochemistry, Japan Scientif. Soc. Press. Tokyo and Martinus Nijhoff/Dr. W. Junk Publ., The Hague, 1983, pp. 193-216.
7. L. Landi, B. Mezzetti, TDZ, auxin and genotype effects on leaf organogenesis in *Fragaria*. Plant Cell Rep., 25: 281-288 (2006).
8. E.C.M. Lee, R.A. de Fossard, Some factors affecting multiple bud formation of strawberry (*Fragaria x ananassa* Duch.) *in vitro*. Acta Hort., 78: 187-196 (1977).
9. G. Ma, W. Xu, Induction of somatic embryogenesis and adventitious shoots from immature leaves of cassava. Plant Cell Tiss. Org. Cult., 70: 281-288 (2002).
10. T. Mori, I.M. Sakura, M. Sekim, S. Furusaki, Use of auxin and cytokinin to regulate anthcyanin production and composition in suspension cultures of strawberry cells. J. Sci. Food Agric., 65: 271-276 (1994).
11. T. Murashige, F. Skoog, A revised medium for rapid growth and bioassays with tobacco tissue cultures. Physiol. Plantarum, 15(3): 473-497 (1962).
12. N.S. Nehra, C. Stushnoff, K.K. Kartha, Direct shoot regeneration from leaf discs. J. Am. Soc. Hort. Sci., 114: 1014-1018 (1989).

13. K. Niemirowicz-Szcytt, The result of intergeneric pollination of *Fragaria x ananassa* Duch. and *Fragaria virginiana* Duch. by *Potentilla* species. Acta Soc. Bot. Pol., 53: 443-454 (1984).
14. A.J. Passey, K.J. Barrett, D.J. James, Adventitious shoot regeneration from seven commercial strawberry cultivars (*Fragaria x ananassa* Duch.) using a range of explant types. Plant Cell Rep., 21: 397-401 (2003).
15. G.C. Phillips, *In vitro* morphogenesis in plants - recent advances. *In Vitro* Cell. Dev. Biol. Plant, 40: 342-345 (2004).
16. R.L.M. Pierik, *In vitro* culture of higher plants as a tool in the propagation of horticultural crops. Acta Hort., 226:25-40 (1998).
17. A.J. Sayegh, M.J. Hennerty, Intergeneric hybrids of *Fragaria* and *Potentilla*, Acta Hort., 348: 151-154 (1993).
18. J.G. Schaart, M.J. Salentijn, F.A. Krens, Tissue specific expression of the ß-glucuronidase reporter gene in transgenic strawberry (*Fragaria x ananassa*) plants. Plant Cell Rep. 21: 313–319 (2002).
19. S. Sorvari, S. Ulvinen, T. Hieteranta, H. Hiirsalmi, Preculture medium promotes direct shoot regeneration from micropropagated strawberry leaf disks. HortScience, 28(1): 55-57 (1993).
20. N.T.P. Thao, Y. Ozaki, H. Okubo, Callus induction and plantlet regeneration in ornamental *Alocasia*. Plant Cell Tiss. Org. Cult., 89: 83-90 (2003).
21. Q. Yonghua, Z. Shanglong, S. Asghar, Z. Lingxiao, Q. Qiaoping, C. Kunsong, X. Changjie, Regeneration mechanism of Toyonoka strawberry under different color plastic films. PlantSci., 168:1409-1424 (2005).
22. 22. M.H. Yu, Callus induction and differentiation from leaf explants of different species of the genus *Beta*. Crop Sci., 29: 205-209 (1989).
23. Y. Zhao, Q. Liu, R.E. Davis, Transgene expression in strawberries driven by heterologous phloem-specific promoter. Plant Cell Rep., 23:224–230 (2004).