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BRIEF COMMUNICATION

An assessment of genetic fidelity of micropropagated plants of *Chlorophytum borivilianum* using RAPD markers

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Abstract

Rapid micropropagation was achieved in *Chlorophytum borivilianum* Santapau and Fernandes using shoot base as explants. Multiple shoots were induced on Murashige and Skoog's (MS) medium supplemented with 3.0 mg dm^{-3} 6-benzylaminopurine, 0.1 mg dm^{-3} 1-naphthaleneacetic acid, 150 mg dm^{-3} adenine sulphates and 3 % saccharose. Rooting was readily achieved upon transferring the shoots onto half strength MS medium supplemented with 0.1 mg dm^{-3} indolebutyric acid and 2 % saccharose. Micropropagated plantlets were hardened in the greenhouse and successfully established in soil. Random amplified polymorphic DNA (RAPD) markers were used to evaluate the genetic stability of the micropropagated plants. Thirty one arbitrary decamers were used to amplify genomic DNA from *in vitro* and *in vivo* plant material to assess the genetic stability. All RAPD profile analysis from micropropagated plants was genetically similar to mother plants.

Additional key words: genetic stability, *in vitro*, medicinal plant, polymerase chain reaction.

Medicinal plants are of great interest to the researchers in the field of biotechnology as most of the drug industries in part depend on plants for the production of pharmaceutical compounds (Rout *et al.* 2000). *Chlorophytum borivilianum* Santapau and Fernandes belongs to the family *Liliaceae* and its common name is safed musli. Its peeled and dried fasciculated roots are considered a wonder drug in traditional Indian medicine, however, excessive collections from its natural stands and destructive harvesting techniques coupled with poor seed germination and low vegetative multiplication ratio have made this species endangered (Maiti and Geetha 2005a) and simultaneously provided the justification for its *in vitro* propagation. Clonal multiplication of *C. borivilianum* through multiple shoot induction has been reported (Purohit *et al*. 1994).

Generally, micropropagated plants from the preformed structures such as shoot tips and axillary buds maintain clonal fidelity (*e.g*. Ostray *et al*. 1994). However, the possibility of generating somaclonal variations still exists (Rani and Raina 2000, Bindiya and Kanwar 2003). Occurrence of somaclonal variation is a potential drawback when the propagation of an elite species is intended due to uncontrollable and unpredictable nature of variation (Rahman and Rajora 2001). On the other hand, stable somaclonal variations of specific type might be advantageous for the improvement of certain traits (Karp 1995, Jain *et al*. 1998) such as disease resistance, high yield, early flowering, and quality of secondary metabolites. A number of molecular markers can be used to assess the genetic fidelity of *in vitro* derived clones. However, random amplified polymorphic DNA (RAPD) is efficient and cheapest tool for the detection of genetic variability in plants (Fernando 1996, Cassells *et al.* 1997, Hussain *et al*. 2008). In this paper, we report on the rapid micropropagation of *C. borivilianum* through shoot bud culture and the genetic homogeneity of established clonal lines, using RAPD markers.

A high yielding line of *C. borivillianum* (registration No. INGR-04113; Maiti and Geetha 2005b) selected on the basis of root yield was used as the initiation plant

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Abbreviations: Ads - adenine sulphate; BA - 6-benzylaminopurine; bp - base pair; IAA - indole-3-acetic acid; IBA - indolebutyric acid; Kin - kinetin; NAA - 1-naphthaleneacetic acid; MS medium - Murashige and Skoog (1962) medium; PCR - polymerase chain reaction; RAPD - random amplified polymorphic DNA; CTAB - cetyltrimethyl ammonium bromide.

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material. Stem discs containing sprouting buds (3 - 4 mm long) were collected from the material stored in perforated polybags at a cool place at the National Research Centre for Medicinal and Aromatic Plants in Anand, Gujarat, India. The explants were washed with 2 % (v/v) detergent *Teepol* (*Qualigen*, Bombay, India) for 20 min, 5 - 6 times rinsed with running tap water, disinfected in a mixture of 2 % (m/v) carbendazim and 2 % (m/v) streptomycin for 3 h, surface sterilized with 0.2 % (m/v) aqueous mercuric chloride solution for 30 min and further rinsed with sterile double distilled water 3 - 4 times. Then the small root portions with shoot buds were cut and these were cultured asceptically in Murashige and Skoog (1962; MS) medium supplemented with different concentrations and combinations of 6-benzylaminopurine (BAP) or kinetin (Kin) at 0.0, 2.0, 2.5 and 3.0 mg dm^{-3}, adenine sulphate (Ads; 0, 50, 100 and 150 mg dm^{-3}) and indole acetic acid (IAA; 0.0, 0.01, 0.05 and 0.1 mg dm^{-3}) or α-naphthalene acetic acid (NAA; 0.0, 0.01, 0.05, and 0.1 mg dm^{-3}) for bud proliferation and multiplication. For root induction, microshoots (2 - 3 cm long) were cultured on half-strength MS basal medium with different concentrations of IAA, IBA or NAA (0.0, 0.1, 0.25 and 0.5 mg dm^{-3}) and 1 % (m/v) saccharose. The pH of the media was adjusted to 5.8 using 0.1 M NaOH or HCl before autoclaving. The MS medium was gelled with 0.8 % (m/v) agar (*Qualigen*). Routinely, 25 cm^3 of molten media was dispensed in to culture tubes (25 × 150 mm), plugged with non-absorbent cotton wrapped in one layer of cheesecloth and sterilized at 121 ºC and 1.06 kg cm^{-2} pressure for 15 min. All cultures were incubated at temperature of 25 ± 2 °C and irradiance of 55 μmol m^{-2} s^{-1} (cool, white fluorescent lamps; 16-h photoperiod). Cultures were subcultured at 4-week intervals onto fresh media with the same composition. Rooted plantlets were removed from culture vessels, washed thoroughly with running tap water and planted in small polybags (6 × 6 cm) containing a mixture of sand, soil and decomposed cow dung in the ratio of 1:1:1 (v/v/v). The plantlets were kept in a greenhouse for 2 weeks for acclimatization before transfer to the field.

DNA was extracted from fresh leaves derived from both fifteen micropropagated and one field-grown mother plants by the cetyltrimethyl ammonium bromide (CTAB) method (Bousquet *et al*. 1990). Fresh leaves (~ 300 mg) were ground to a powder in liquid nitrogen. The powder was transferred to a 50 cm^3 sterile polyproplyne tube containing 10 cm^3 of extraction buffer contained 2 % (m/v) CTAB, 1.4 M NaCl, 20 mM EDTA, 100 mM Tris-HCl, pH 8.0 and 0.2 % (v/v) β-mercaptoethanol. The homogenate was incubated at 65 °C for 1 h, extracted with an equal volume of chloroform: isoamyl alcohol (24:1), and centrifuged (*Sorvall Super T21*, Kendro, Germany) at 12 410 *g* for 20 min. The DNA was precipitated from the aqueous phase by mixing with an equal volume of isopropanol followed by washing with 70 % ethanol. The DNA pellet was then air dried and resuspended in 10 mM Tris, 0.1 M EDTA buffer (pH 8.0). DNA quantity was estimated spectrophotometrically (*Cary 300*, *BioVarian*, Mulgrave, Australia) by measuring the absorbance at 260 nm. Sixty arbitrary 10-base primers (*Operon Technologies*, Almeda, USA) were used for polymerase chain reaction (PCR). Amplification reactions were performed in 0.025 cm^3 volume contained 0.0025 cm^3 of 10× assay buffer (100 mM Tris-HCl; pH 8.3, 500 mM KCl, 15 mM $MgCl_2$ and 0.1 % gelatin), 200 μM dNTPs (dATP, dCTP, dGTP and dTTP) (*MBI Ferment*, Glen Burnie, USA) 15 ng of primer, 1.0 unit of Taq DNA polymerase (*Bangalore Genei*, Bangalore, India) and 30 ng of template DNA. The amplification reaction was carried out in a DNA thermal cycler (*Eppendorf AG*, Hamburg, Germany) programmed for 44 cycles as follows: 1st cycle of 5 min at 94 ºC followed by 43 cycles each of 1 min at 92 ºC, 1 min at 37 ºC, 2 min at 72 ºC. The final step consisted of one cycle of 7 min at 72 ºC for complete polymerization. The soak temperature was 4 ºC. After completion of the PCR, 0.0025 cm^3 of 6× loading dye (*MBI Ferment*) was added to the amplified products and were electrophoresed

Table 1. Description of 31 decamer primers used for fingerprint analysis of micropropagated plants and the source plant of *Chlorophytum borivilianum.*

No.	Primer	Sequence	Amplified fragments	Fragment size [bp]
1	OPA-01	5'CAGGCCCTTC3'	7	700-2880
2	OPA-06	5'GGTCCCTGAC3'	8	640-3000
3	OPA-08	5'GTGACGTAGG3'	5	620-1880
4	OPA-09	5'GGGTAACGCC3'	7	325-1500
5	OPA-10	5'GTGATCGCAG3'	5	600-2000
6	OPA-14	5'TCTGTGCTGG3'	7	500-1844
7	OPA-15	5'TTCCGAACCC3'	5	1020-3000
8	OPA-16	5'AGCCAGCGAA3'	8	380-1917
9	OPA-17	5'GACCGCTTGT3'	5	600-2300
10	OPA-19	5'CAAACGTCGG3'	6	466-1500
11	OPA-20	5'GTTGCGATCC3'	5	450-1380
12	OPC-01	5'TTCGAGCCAG3'	5	590-1200
13	OPC-03	5'GGGGGTCTTT3'	4	530-1080
14	OPC-08	5'TGGACCGGTG3'	4	715-2000
15	OPC-15	5'GACGGATCAG3'	4	333-1500
16	OPD-20	5'ACCCGGTCAC3'	5	520-2600
17	OPJ-05	5'CTCCATGGGG3'	5	515-2440
18	OPJ-07	5'CCTCTCGACA3'	6	600-2000
19	OPJ-09	5'TGAGCCTCAC3'	5	680-1355
20	OPJ-11	5'ACTCCTGCGA3'	5	520-2133
21	OPJ-13	5'CCACACTACC3'	6	700-2033
22	OPN-01	5'CTCACGTTGG3'	4	725-2000
23	OPN-05	5'ACTGAACGCC3'	6	400->3000
24	OPN-09	5'TGCCGGCTTG3'	4	510-2000
25	OPN-10	5'ACAACTGGGG3'	6	375-1450
26	OPN-11	5'TCGCCGCAAA3'	6	1000-2375
27	OPN-13	5'AGCGTCACTC3'	5	855-2560
28	OPN-15	5'CAGCGACTGT3'	6	600-2000
29	OPN-20	5'GGTGCTCCGT3'	5	600->3000
30	OPP-06	5'GTGGGCTGAC3'	4	775->3000
31	OPP-08	5'ACATCGCCCA3'	4	640-1312

in a 1.5 % (m/v) agarose gels (*MBI Ferment*) with 1× TAE buffer, stained with ethidium bromide and photographed under UV radiation. The sizes of the amplification products were estimated by comparing them to standard DNA ladder (*MBI Ferment*). All the reactions were repeated three times. Eight primers were amplified on the basis of the clarity of the banding patterns. Amplified DNA markers were scored as present or absent for both the micropropagated and the mother plants. Electrophoretic DNA bands of low visual intensity that could not be readily differentiated as present or absent were considered ambiguous markers and were not scored.

Fifteen cultures were used per treatment and each experiment was repeated three times. All cultures were observed periodically and occurrence of any morphological changes was recorded by visual observations. The data were analyzed statistically using Duncan's multiple range test (DMRT) in *MSTAC Vr. 2.10*.

Of the two cytokinins used, BAP was more effective for shoot proliferation than Kin. Rapid multiplication of shoots was achieved on ½ MS medium with 3.0 mg dm^{-3} BAP, 0.1 mg dm^{-3} NAA and 3 % (m/v) saccharose within 4 weeks of culture. Addition of 150 mg dm^{-3} Ads enhanced the rate of shoot multiplication. Among three auxins used, IBA was more effective for root induction than NAA and IAA. Rooting was totally inhibited on the medium devoid of auxins. The root initiation took place within 8 - 10 d of culture on ½ MS medium supplemented with 0.1 mg dm^{-3} IBA with 2 % saccharose. The percentage of shoots forming roots and the number of roots/shoot varied significantly with different concentrations of IAA, IBA and NAA. Rooted plantlets acclimatized in the greenhouse for 2 weeks and 93 - 95 % of transferred plants to the field survived. At this point, the plants grew normally and no gross morphological variation was noticed.

Of the hundred random primers tested, thirty one were selected on the basis of their clarity in the electrophoresis banding patterns. The results showed that the primers OPA-01, OPA-06, OPA-08, OPA-09, OPA-10, OPA-14, OPA-15, OPA-16, OPA-17, OPA-19, OPA-20, OPC-01, OPC-03, OPC-08, OPC-15, OPD-20, OPJ-05, OPJ-07, OPJ-09, OPJ-11, OPJ-13, OPN-01, OPN-09, OPN-10,

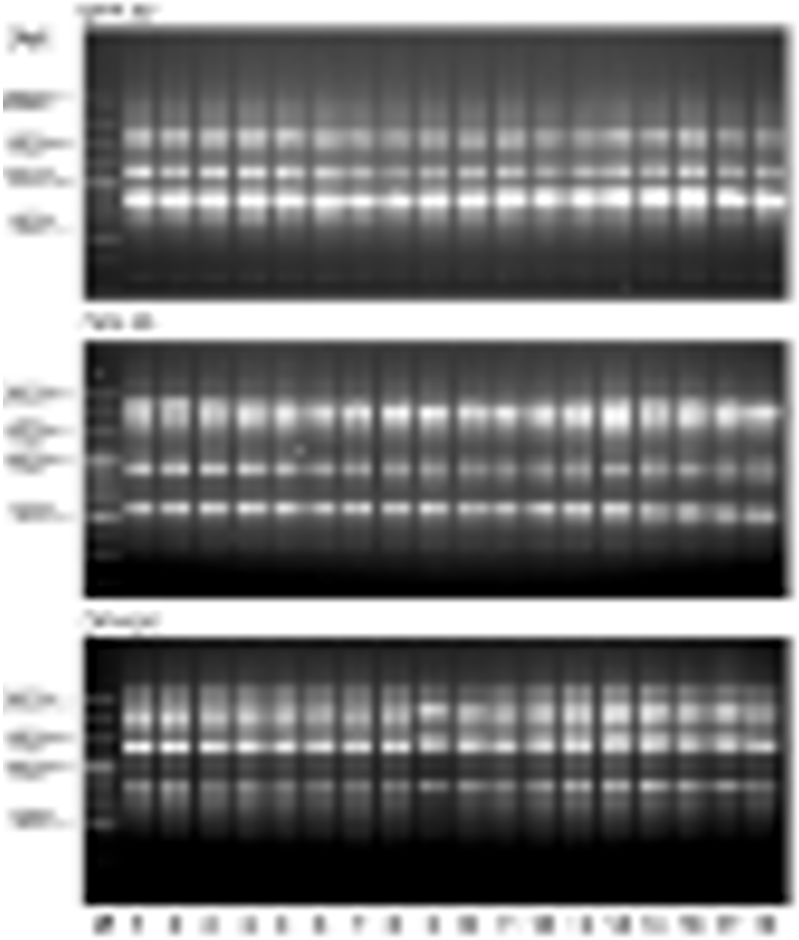

Fig. 1. RAPD profiles of micropropagated and field grown mother plants of *C.borivilianum* generated by primers OPA-17, OPJ-15 and OPN-20. *Lane 1* shows RAPD bands from the mother plant and *lanes 2 - 18* indicate the micropropagated plants. *M* indicates the marker.

OPN-11, OPN-13, OPN-15, OPN-20, OPP-06 and OPP-08 produced amplification products, which were monomorphic across all micropropagated plants. No polymorphism was detected in the micropropagated plants (Table 1). The size of the band produced by OPA-17, OPJ-05 and OPN-20 primers ranged from 600 to 2300 bp, 515 to 2440 bp and 600 bp to >3000 bp, respectively (Fig. 1). Micropropagated plants derived from shoot tips and axillary buds have been previously reported to maintain clonal stability (Ahuja 1987, Ostray *et al*. 1994). However, occasionally there is still a chance of obtaining somaclonal variation through adopting this approach (Rani and Raina 2000). The genetic stability of *C. borivilianum* shown in our study is an agreement with Rout *et al*. (1998) who reported that micropropagated plants derived from the shoot buds of ginger did not show any genetic variation following RAPD analysis. It is thought that genetic stability is maintained when meristem culture is utilized for micropropagation purposes because organized meristems are generally more resistant to genetic changes that might occur during cell division and differentiation *in vitro* (Shenoy and Vasil 1992).

In the present study, the bands amplified through RAPD showed monomorphism with the mother plant and were also similar among the *in vitro* raised plants. This study provides the first information on genetic stability detected by RAPD markers of micropropagated a high yielding line of *C. borivilianum*. The RAPD technique (Williams *et al*. 1990) has been extensively used for studying genetic fidelity of *in vitro* raised plants in a number of species (Kawiak and Lojkowska 2004, Hembrom *et al*. 2006, Tyagi *et al*. 2007, Yang *et al*. 2008). This method often yields large number of markers; it is technically simple to perform and does not need either large amounts of DNA or previous information of DNA sequences, which are often unknown in many plants. Moreover, RAPD analysis is comparatively easy, cost effective and requires comparatively minimum time and also involves no radioactive compound to be handled. Employing RAPD technique, various authors have reported the absence of genetic variation in many species such as *Picea marina*, *Festuca pratensis*, *Liriodendron tulipifera* (Merkle *et al*. 1988, Isabel 1993, Valles *et al*. 1993). Similarly, some investigators have also reported polymorphism in micropropagated plants through RAPD (Munthali *et al*. 1996, Major *et al*., 1998, Bindiya and Kanwar 2003). Rani *et al*. (1995) found variations among micropropagated *Populus deltoides* plants originating from the same clone though they were morphologically similar. Additionally, intraclonal polymorphisms amongst micropropagated *Begonia* plants were also observed and these did not correlate with the different phenotypes evaluated (Bouman *et al*. 1992).

In conclusion, an efficient protocol for mass propagation of *C. borivilianum* was developed. Our results also suggest that RAPD markers can be successfully used to assess genetic variations in *C. borivilianum* micropropagated plants.

References

Ahuja, M.R.: *In vitro* propagation of poplar and aspen. - In: Bonga, J.M., Durzan, D.J. (ed.): Cell and Tissue Culture in Forestry. Vol. 3. Pp. 207-223. Martinus Nijhof, Dodrecht 1987.

Bindiya, K., Kanwar, K.: Random amplified polymorphic DNA (RAPDs) markers for genetic analysis in micropropagated plants of *Robinia pseudoacacia* L. - Euphytica **132**: 41-47, 2003.

Bouman, H., Kujipers, A. M., De Klerk, G.J.: The influence of tissue culture methods on somaclonal variation in *Begonia*. - Physiol. Plant. **85**: 39-45, 1992.

Bousquet, J., Simon, L., Lalonde, M.: DNA amplification from vegetative and sexual tissues of trees using polymerase chain reaction. - Can. J. Forest Res. **20**: 254-257, 1990.

Cassells, A.C., Croke, J.T., Doyle, B.M.: Evaluation of image analysis, flow cytometry, and RAPD analysis for the assessment of somaclonal variation and induced mutation in tissue culture-derived *Pelargonium* plants.- Angew. Bot. **71**: 125-130, 1997.

Fernando, A., Barney, V.E., Tohme, J., Roca, W.M.: Stability of cassava plants at the DNA level after retrieval from 10 years of *in vitro* storage. - Euphytica **90**: 307-313,1996.

Hembrom, M.E., Martin, K.P., Patchathundikandi, S.K., Madassery, J.: Rapid *in vitro* production of true-to-type plants of *Pogostemon heyneanus* through dedifferentiated axillary buds. - *In Vitro* cell. dev. Biol. Plant. **42**: 283-286, 2006.

Hussain, Z., Tyagi, R.K., Sharma, R., Agrawal, A.: Genetic diversity in *in vitro*-conserved germplasm of *Curcuma* L. as revealed by RAPD markers. - Biol. Plant. **52**: 627-633, 2008.

Isabel, N.L., Tremblay, M., Michaud, F., Tremblay, M., Bousquet, J.: RAPDs as an aid to evaluate the genetic integrity of somatic embryogenesis derived population of *Picea mariana* (Mill.) B.S.P. - Theor. appl. Genet. **86**: 81-87, 1993.

Jain, S.M., Brar D.S., Ahloowalia B.S. (ed.): Somaclonal Variation and Induced Mutations in Crop Improvement. - Kluwer Publishers, Dordrecht 1998.

Karp, A.: Somaclonal variation as a tool for crop improvement. - Euphytica **85**: 295-302, 1995.

Kawiak, A., Lojkowska, E.: Application of RAPD in the determination of genetic fidelity in micropropagated *Drosera* planlets. - *In Vitro* cell. dev. Biol. Plant. **40**: 592-595, 2004.

Maiti, S., Geetha, K.A.: Plant introduction in medicinal and aromatic plants: achievements and opportunities. - Indian J. Plant Genet. Resour. **18**: 22-23, 2005a.

Maiti, S., Geetha, K.A.: Characterization, genetic improvement and cultivation of *Chlorophytum borivilianum* - an important medicinal plant of India. - Plant Genetic Resour. **3**: 264-272, 2005b.

Major, A., Malvolti, M.E., Cannata, F.: Comparison of isozyme and RAPD variability of black locust (*Robinia pseudoacacia*) clones selected for silvicultural objectives. - J. Genet. Breed. **52**: 49-62, 1998.

Merkle, S.A., Chou, P.L., Sommer, H.E.: Stability of highly repeated sequences in the DNA of embryogenic cultures of yellow poplar. - In: Cheliak, W.M., Yapa, A.C. (ed.): Molecular Genetics of Forest Trees. P. 80. Petawawa National Forestry Institute, Chalk River 1988.

Munthali, M.T., Newbury, H.J., Ford-Lloyd, B.V.: The detection of somaclonal variants of beet using RAPD. - Plant Cell Rep. **15**: 744-748, 1996.

Murashige, T., Skoog, F.: A revised medium for rapid growth and bioassays with tobacco tissue culture. - Physiol. Plant. **15**: 473-479, 1962.

Ostray, M.E., Hackett, W., Michler, C., Serres, R., McCown, B.: Influence of regeneration method and tissue culture on the frequency of somatic variation in *Populus* to infection by *Septoria musiva*. - Plant Sci. **97**: 209-215, 1994.

Purohit. S.D., Dave, A., Kukda, G.: Micropropagation of safed musli (*Chlorophytum borivilianum*), a rare Indian medicinal herb. - Plant Cell Tissue Organ Cult. **39**: 93-96, 1994.

Rahman, M.H., Rajora, O.P.: Microsatellite DNA somaclonal variation in micropropagated trembling aspen (*Populus tremuloides*). - Plant Cell Rep. **20**: 531-536, 2001.

Rani, V., Raina, S.N.: Genetic fidelity of organized meristem derived micropropagated plants: a critical reappraisal. - *In Vitro* cell. dev. Biol. Plant **36**: 319-330, 2000.

Rani, V., Parida, A., Raina, S.N.: Random amplified polymorphic DNA (RAPD) markers for genetic analysis in micropropagated plants of *Populus deltoides* Marsh. - Plant Cell Rep. **14**: 459-462, 1995.

Rout, G.R., Das, P., Goel, S., Raina, S.N.: Determination of genetic stability of micropropagated plants of ginger using random amplified polymorphic DNA (RAPD) markers. - Bot. Bull. Acad. sin. **39**: 23-27; 1998.

Rout, G.R., Samantaray, S., Das, P.: *In vitro* manipulation and propagation of medicinal plants. - Biotechnol. Adv. **18**: 91-120, 2000.

Shenoy, V.B., Vasil, I.K.: Biochemical and molecular analysis of plants derived from embryogenic tissue cultures of napier grass (*Pannisetum purpureum* K. Schum). - Theor. appl. Genet. **83**: 947-955, 1992.

Tyagi, R.K., Agarwal, A., Mahalakshmi, C., Hussain, Z., Tyagi, H.: Low-cost media for *in vitro* conservation of turmeric (*Curcuma longa* L.) and genetic stability assessment using RAPD markers. - *In Vitro* cell. dev. Biol. Plant. **43**: 51-58, 2007.

Valles, M.P., Wang, Z.Y., Montavon, P., Potrykus, I, Spangenberg, G.: Analysis of genetic stability of plants regenerated from suspension cultures and protoplasts of meadow fescue (*Festuca pratensis* Huds.). - Plant Cell Rep. **12**: 101-106, 1993.

Williams, J.G.K., Kubelik, A.R., Livak, K.J., Rafalski, J.A., Tingey, S.V.: DNA polymorphism amplified by arbitrary primers are useful as genetic markers. - Nucl. Acids Res. **18**: 6531-6535, 1990.

Yang, X.M., An, L.Z., Xiong, Y.C., Zhang, J.P., Li, Y., Xu, S.J.: Somatic embryogenesis from immature zygotic embryos and monitoring the genetic fidelity of regenerated plants in grapevine. - Biol. Plant. **52**: 209-214, 2008.