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IN VITRO PROPAGATION OF *SILYBUM MARIANUM* (L.) GAERTN. AND GENETIC FIDELITY ASSESSMENT OF MICROPROPAGATED PLANTS

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Abstract

Silybum marianum (milk thistle), an annual and biennial herbaceous plant, has been used to treat liver disease for 2000 years. An efficient system for micropropagation from leaf explants of wild-grown *S. marianum* was successfully established and the genetic fidelity of micropropagated plants was assessed using PCR-based random amplified polymorphic DNA (RAPD). The highest frequency (98.9%) of callus induction was obtained from explants cultivated for 4 weeks on Murashige and Skoog (MS) medium supplemented with 1.5 mg l^{-1} N^6-Benzylaminopurine (BA) and 2.0 mg l^{-1} α-Naphthaleneacetic acid (NAA). The optimal medium for shoots organogenesis was MS supplemented with 3.0 mg l^{-1} Gibberellic acid (GA_3) and 1.0 mg l^{-1} NAA, while shoot proliferation was accomplished in MS supplemented with 1.0 mg l^{-1} BA and 0.3 mg l^{-1} NAA. Rooting (94.5%) of these shoots was achieved after 4 weeks on $^1/_2$ MS medium supplemented with 1.0 mg l^{-1} IBA. A total of 92% of the plants were surviving 6 weeks after transplantation of plantlets to soil. RAPD analysis revealed that the genetic fidelity between the parent and the *In vitro*-raised plantlets exhibited 100% similarity. This confirmed the true-to-type nature of the *In vitro*-raised clones.

Key words: *Silybum marianum*. Micropropagation. Genetic fidelity. PCR-based random amplified polymorphic DNA (RAPD), Plant growth regulators (PGRs).

Introduction

Silybum marianum (milk thistle) is an annual and biennial herbaceous plant belonging to the Asteraceae family. Originally a native of the Mediterranean basin, *S. marianum* is now found throughout the world (Khan *et al*., 2014). *S. marianum* has been used to treat liver disease as a hepatoprotective herb since 2000 years ago. The chemical compounds with medicinal value are found in the seeds of the plant. Seeds of milk thistle contain significant amounts of silymarin which is a mixture of flavonolignans such as silychristin, silydianin, silybin, and isosilybin, taxifolin (Engelberth *et al.*, 2008; Wallace *et al.*, 2005; Kurkin *et al.*, 2001). The hepatoprotective effect of silymarin is mediated through different mechanisms, including antioxidant and free radical scavenging activities (Shaker *et al.*, 2010), and hepatocellular membrane stabilization (Basiglio *et al.*, 2009) and cell permeability regulation, anti-inflammatory activities (Gupta *et al.*, 2000), liver regeneration stimulation and inhibition of collagen fibers deposition. In addition to treating liver disorders, silymarin has beneficial property on a wide variety of other disorders, such as anticancer effect (Zi *et al.*, 1997; Chatterjee *et al.*, 1996; Öztürk *et al.*, 2015), prevention of hemolysis of red blood cells (Zou *et al.*, 2001) and renal protection (Rafieian-Kopaie & Nasri, 2012; Soto *et al.*, 2010), hypoglycemic activity and prevention of insulin resistance (Huseini *et al.*, 2006).

At present, the increasing demand for silymarin is endangering the *S. marianum* populations (Ahmad *et al.*, 2008). Moreover, the efficiency of seed germination and seedling growth, which highly depends on various biological and environmental factors, is very low (Shinwari *et al*., 2014). Besides, manual handling of the plants is very difficult because leaves and flowers are spiny and, as the plant is cultivated in rows, using harvesting machine causes damage and reduces the crop yield (Hammouda *et al.*, 1993). Although advanced microbial and chemical methods can synthesize medicinal and aromatic compounds, the cost in many cases is high. To meet the increasing need for plantlets, it is imperative to develop a protocol for mass propagation through tissue culture for the large scale production of *S. marianum*.

Scaling up of any micropropagation protocol is severely hindered due to the incidence of genetic instability and somaclonal variations which occur mostly in response to the stresses imposed on the plant under *In vitro* conditions (Haisel *et al.*, 2001). Accordingly, it is necessary to detect the genetic uniformity of micropropagated plants at an earlier stage of plant growth. RAPD analysis is the simplest, highly sensitive, largely automatable technique which requires only a small amount of random sampling over the whole genome, high levels of polymorphism and does not require the use of radioisotopes (Williams *et al.*, 1990). This technique is efficient and inexpensive and has been widely employed to study the origin, evolution, taxonomic, and genetic diversity of species, phylogenetics, as well as for the construction of genetic-linkage maps (Hadrys *et al.*, 1992) and germplasm analysis (Powell *et al.*, 1996).

Until now, most scholars have focused on the active ingredients, pharmacological effects and the cultivation aspect of *S. marianum*, and few studies have been reported on its tissue culture. Actually, although the propagation of *S. marianum* through *In vitro* techniques has been reported by several researchers using different explant sources with regeneration pathways (Cimino *et al.*, 2006, Iqbal & Srivastava, 2000), there are no protocols to check genetic and biochemical fidelity of the regenerated plantlets. Therefore, in this study, the aim was to develop a simple and efficient regeneration protocol for *S. marianum* as well as to investigate genomic stability or variation of callus-regenerated plants using the RAPD analysis technique.

Materials and Methods

Plant material and source of explants: The shoots with apical buds (about 3 to 4 cm from the top) from 1-2-year-old *S. marianum* plants were collected from the garden of Northwest University, Xi'an. The materials were washed in running tap water, then the explants were surface-sterilized with 70% (v/v) ethanol for 30 s, followed by three rinses in distilled water. The final step of surface sterilization was performed with 0.1% mercuric chloride containing a few drops of Tween-80 (poly-oxyethylene sorbitan monolaurate) for 6 min. This is followed by rinsing the explants five times with sterile double-distilled water. Nodal segments were vertically and aseptically implanted on autoclaved MS culture medium for 4 weeks.

Callus induction: The immature leaves were cut into small segments of about 1.0×1.0 cm (length × width) each and cultured in an autoclaved 150ml Erlenmeyer flasks containing 50ml MS medium supplemented with various concentration combinations of BA and NAA for the emergence of callus. The number of explants developing callus was recorded after 4 weeks. A plant growth regulators (PGRs)-free medium was used as control.

Shoot organogenesis and multiplication: The yellowish friable callus was excised, and transferred to MS medium containing different concentration combinations of NAA and BA or GA_3 for shoot organogenesis. In addition, the regenerated shoots were transferred to a different PGRs (BA and NAA) medium for shoot multiplication. Data were collected after 4 weeks from inoculation.

Rooting and acclimatization to soil: Healthy and elongated shoots were cultured on either MS or $^1/_2$ MS medium at different concentrations of NAA or indole-3-butyric acid (IBA). The percentage of shoots with root induction and the length of the root were recorded after 4 weeks. After rhizogenesis, healthy plantlets with well-developed roots were removed from the medium and washed thoroughly in sterile-distilled water to remove MS medium attached to the roots. The rooted plantlets were treated with 1% Bavistin solution to prevent any fungal infection before being transferred to small disposable plastic pot containing soil: sand (1:1,v/v), acclimatized, and grown under greenhouse conditions maintained at 25±2ºC with 16-h/day photoperiod and covered with transparent plastic bags to maintain a relative humidity range of 70 to 80 %. The survival rate of transplanted plantlets was determined after 6 weeks.

Culture conditions: MS medium supplemented with 30 g l^{-1} sucrose and 7 g l^{-1} agar was used as basal medium throughout the experiment. Different PGRs were added to the MS basal medium and the pH of all medium was adjusted to 5.8 to 6.2 using KOH or HCl, prior to autoclaving at 1.05 kg/cm^2 pressure at 121ºC for 20 min. In all experiments, a PGRs-free medium was used as control. The cultures were maintained at 25±2ºC under a 16-h photoperiod with light intensity of 35-40 µmol $m^{-2}s^{-1}$ (cool white fluorescent tubes). Each treatment consisted of 30 repetition and all the experiments were repeated three times and were performed under uniform culture conditions.

DNA isolation and RAPD analysis: For genetic fidelity evaluation, four hardened tagged regenerated plants were randomly chosen along with the single donor parent plant used for RAPD analysis. Total genomic DNA was extracted from young leaf tissue (0.2 g) by using the modified cetyl trimethyl ammonium bromide (CTAB) method (Khan *et al.*, 2007). The total DNA was quantified and its quality was verified by monitoring the $^{A260}/_{A280}$ absorbance ratio using the Biospec-nano (Shimadzu, Tokyo, Japan). The integrity of the DNA sample was tested by 1.0 % agarose gel electrophoresis and ethidium bromide (EB) staining. Finally, each DNA sample was diluted and normalized to a concentration of 50 ng µl^{-1}. A set of 40 decamer RAPD primers (Sangon, shanghai, China) were screened for their repeatable amplification with the DNA from the aforesaid plants including the elite parent plant to assess the genetic stability of the regenerations. A total of 20 decamer RAPD primers were finally selected for the analysis on the basis of their amplification products for clear and scorable banding patterns. RAPD-PCR was carried out in a total volume of 25µl containing 30 ng of genomic DNA, 12.5 µl master mix (Tiangen, Beijing, China), 0.4µl (20µM) decamer primer, and sterile water up to 25 µl. The RAPD amplification reactions were performed in a thermocycler (Eppendorf AG, Hamburg, Germany). The amplification conditions, using RAPD primers, were as follows: initial DNA denaturation at 94°C for 5 min, followed by 40 cycles of 1 min denaturation at 94°C, 1 min annealing at 35 °C, 1.5 min of extension at 72°C, and a final extension at 72°C for 10 min. The RAPD amplified fragments were subsequently separated on a 1.0% agarose gel by electrophoresis in 1×Tris-acetate-EDTA (TAE) buffer for 40 min at 130 V. The gel was stained with EB and bands were visualized using a gel documentation system (Tanon, shanghai, China).

Recording of data and statistical analysis: For *In vitro* regeneration experiments, the average multiplication rate of shoots per node, the average number of shoots per explant, the average shoot length, the average number of roots per shoot and the average root length were calculated and represented as mean values along with standard error (SE). The data expressed as mean ± SE were statistically analyzed by ANOVA (Analysis of Variance) using the Statistical Package for Social Sciences (SPSS) version 20.0. The differences between means were tested for significance by Duncan's multiple range test (DMRT) at $p<0.05$. The RAPD bands were scored as present (1) or absent (0), each of which was treated as an independent character regardless of its intensity. Only the prominent and reproducible bands obtained for each RAPD primer were considered. Faint or unclear bands were disregarded.

Results

Callus induction: The surface-sterilization treatment was efficient, as indicated by the lower contamination observed in the culture. In the preliminary work, BA or NAA alone produced low rate of callus induction, and callus formation was lowest with BA (data not show). On the other hand, addition of NAA significantly promoted callus induction. Varied response of callus induction was observed with different concentration combinations of BA and NAA (Table 1). The size of the piece of immature leaf was generally enlarged after two weeks of induction with the incision margin of the explants surrounded by white and loose callus particles. Four weeks later, a larger number of callus were observed on the outer surface of the explants (Fig. 1a). The highest frequency of callus induction was observed on basal medium supplemented with 1.5 mg l^{-1} BA and 2.0 mg l^{-1} NAA, when 98.9% of the cultured immature leaf piece produced callus (Fig. 1a; Table 1). No callus was observed when explants were incubated on MS basal medium.

Table 1. Effect of various concentrations of BA with NAA on callus induction from immature leaf explants of *S. marianum*.

Plant growth regulators (mg/l)		Inoculation number	Percent callusing (%)
BA	NAA		
1.0	0.5	30	46.7 ± 3.4
1.0	1.0	30	58.9 ± 5.1
1.0	2.0	30	81.1 ± 3.8
1.5	0.5	30	55.6 ± 5.1
1.5	1.0	30	86.7 ± 6.7
1.5	2.0	30	98.9 ± 1.9
2.0	0.5	30	50.0 ± 3.3
2.0	1.0	30	84.3 ± 5.1
2.0	2.0	30	88.7 ± 5.1

Mean values within a column followed by the same letter are not significantly different by Duncan's multiple range test at $P<0.05$. Culture period 4 weeks. Values correspond to means ± SE.

Shoot organogenesis and multiplication: Shoot organogenesis from immature leaf-derived callus was observed on MS medium supplemented with different combinations of NAA with BA or GA_3. The small shoot clumps on callus surface were observed following a week of cultivation. After 15 days of cultivation, almost all the callus surfaces were covered with green buds (Fig. 1b). Subsequently, small shoots were produced (Fig. 1c). Among the combinations tested, the 3.0 mg l^{-1} GA_3 and 1.0 mg l^{-1} NAA combination produced an optimum of 95.6% response with 5.5 shoots per g callus (Table 2). The regenerated shoots were transferred to shoot propagation medium containing various combinations of BA and NAA. The combination of 1.0 mg l^{-1} BA and 0.3 mg l^{-1} NAA on MS medium generated the best response for shoots multiplication with an average multiplication rate of 6.6 and an average shoot length of 3.1 cm after 4 weeks of culture (Fig. 1d; Table 3). Basal medium did not support induction of shoot organogenesis and multiplication.

Rooting and field transfer: Well-developed shoots were used for root induction studies on MS medium with various supplements. The shoots were cultured on $^1/_2$ MS or MS medium with NAA or IBA. Root induction on $^1/_2$ MS or MS basal medium was treated as control. White, healthy roots emerged from the basal region of the shoots after ten days of cultivation. However, the number and length of the roots varied with different culture media (Fig. 1e; Table 4). The maximum number of roots (6.6) was observed on $^1/_2$ MS medium containing 1.0 mg l^{-1} of IBA, with an average root length of 3.2 cm. A total of 50 plantlets with roots were transplanted into plastic pots (Fig. 1f), 46 (92%) of which survived. Additionally, no morphology variation was found compared with the parent plant.

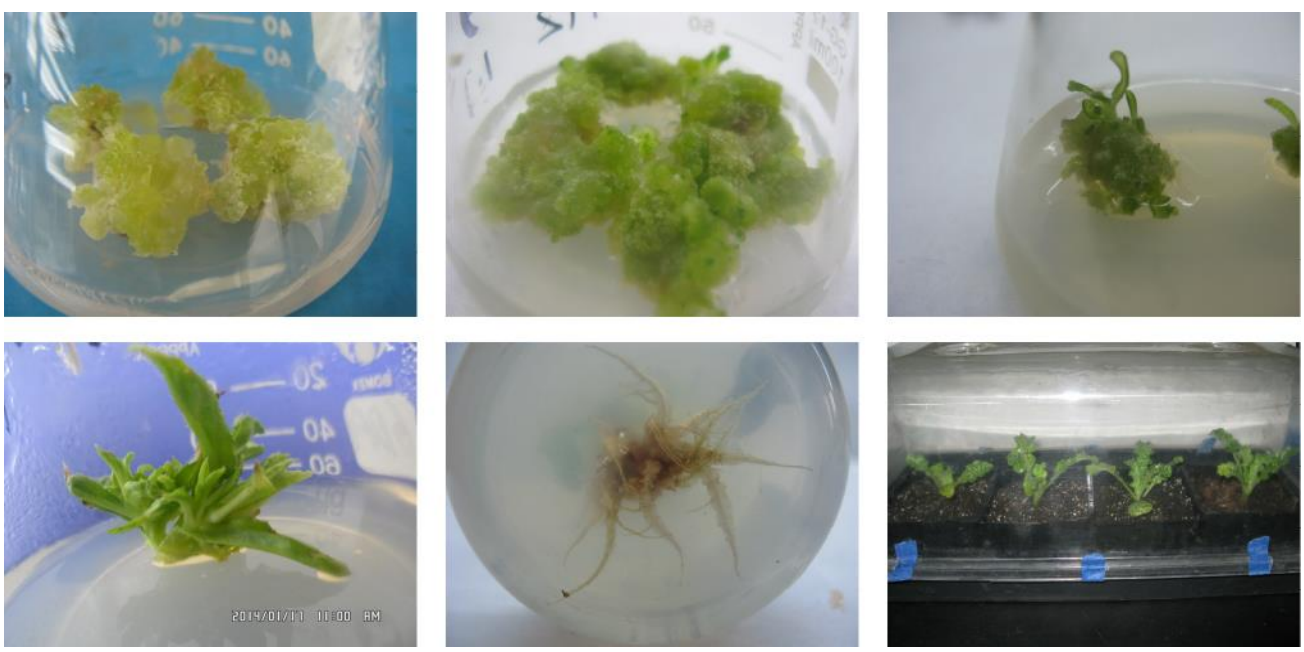

Fig. 1. Different stage of regeneration of *Silybum marianum* **a.** Callus formation from leaf explant, **b.**Occurrence of shoot clumps, **c.** Shoot organogenesis from leaf explant, **d.** Proliferation and elongation of regenerated shoots, **e.** Rooting of regenerated shoots, **f.** Acclimatization plants in bags.

Table 2. Effect of various concentrations of NAA and GA_3 or BA on *In vitro* shoot regeneration from the immature leaf-derived callus of *S. marianum*.

Plant growth regulators (mg/l)			Percent callus forming shoots (%)	Mean number of shoots per g callus
GA_3	BA	NAA		
4.5	0	1.5	72.3 ± 39	$3.0 \pm 0.78^{d,e}$
4.5	0	1.0	76.7 ± 5.8	$3.1 \pm 0.93^{e,f}$
4.5	0	0.5	71.1 ± 5.1	$2.6 \pm 0.92^{b,c}$
3.0	0	1.5	87.8 ± 8.4	4.9 ± 0.95^{g}
3.0	0	1.0	95.6 ± 5.1	5.5 ± 1.06^{k}
3.0	0	0.5	87.8 ± 6.9	4.3 ± 1.08^{i}
1.5	0	1.5	80.0 ± 3.3	3.8 ± 0.97^{h}
1.5	0	1.0	84.3 ± 2.0	4.3 ± 1.01^{i}
1.5	0	0.5	81.0 ± 8.5	$3.7 \pm 0.88^{g,h}$
1.0	0	1.5	71.1 ± 1.9	$2.9 \pm 0.94^{c,d,e}$
1.0	0	1.0	78.9 ± 5.1	$3.4 \pm 1.08^{f,g}$
1.0	0	0.5	75.6 ± 2.0	$3.2 \pm 1.00^{e,f}$
0	4.5	1.5	65.6 ± 8.4	$2.4 \pm 0.85^{a,b}$
0	4.5	1.0	71.1 ± 7.0	$2.6 \pm 0.69^{b,c}$
0	4.5	0.5	66.6 ± 5.8	2.2 ± 0.85^{a}
0	3.0	1.5	83.3 ± 5.8	4.2 ± 0.96^{i}
0	3.0	1.0	86.7 ± 3.3	4.7 ± 1.04^{g}
0	3.0	0.5	82.3 ± 3.9	3.8 ± 0.95^{h}
0	1.5	1.5	74.4 ± 8.4	$3.2 \pm 0.95^{e,f}$
0	1.5	1.0	76.7 ± 6.7	$3.7 \pm 0.95^{g,h}$
0	1.5	0.5	74.5 ± 3.9	$3.1 \pm 1.00^{e,f}$
0	1.0	1.5	65.6 ± 5.0	$2.6 \pm 0.87^{b,c}$
0	1.0	1.0	72.2 ± 8.4	$2.7 \pm 0.83^{b,c}$
0	1.0	0.5	71.3 ± 5.1	2.1 ± 0.73^{a}

Mean values within a column followed by the same letter are not significantly different by Duncan's multiple range test at $p<0.05$. Culture period 4 weeks. Values correspond to means ± SE.

Table 3. Effect of various concentrations of NAA and BA on *In vitro* regeneration from the immature shoot of leaf-derived callus of *S. marianum*.

Plant growth regulators (mg/l)		Mean shoot length (cm)	Multiplication rate
BA	NAA		
0.5	0.3	2.10 ± 0.32^{b}	3.4 ± 0.93^{c}
0.5	0.5	1.91 ± 0.36^{a}	$3.3 \pm 0.88^{b,c}$
0.5	1.0	2.94 ± 0.45^{e}	2.8 ± 0.93^{a}
1.0	0.3	3.10 ± 0.34^{f}	6.6 ± 1.17^{f}
1.0	0.5	2.63 ± 0.37^{c}	5.8 ± 0.96^{e}
1.0	1.0	$2.71 \pm 0.34^{c,d}$	$3.2 \pm 1.05^{b,c}$
2.0	0.3	3.30 ± 0.35^{g}	3.8 ± 1.03^{d}
2.0	0.5	2.80 ± 0.28^{d}	3.4 ± 0.93^{c}
2.0	1.0	$3.00 \pm 0.34^{e,f}$	$3.0 \pm 0.99^{a,b}$

Mean values within a column followed by the same letter are not significantly different by Duncan's multiple range test at $p<0.05$. Culture period 4 weeks. Values correspond to means ± SE.

Assessment of fidelity of the regenerated plants by RAPD analysis: In order to test genetic fidelity, acclimatized micropropagated plants along with the parent plant were assessed by RAPD analysis. Out of the total 40 primers screened, 20 primers could produce the clear and reproducible scorable bands and were selected for further study (Table 5). The number of bands for each primer set varies from 1 to 9 with an average of 3.75 bands per primer set. All the banding profiles generated by RAPD analysis of micropropagated plants and parent plant are shown (Fig. 2). No polymorphic bands were observed. RAPD analysis revealed no evidence of genetic variation either within or between the plants derived from calluses and the parent plant.

Discussion

Development of a protocol for micropropagation of field-grown plants through tissue culture is one of the important requisitions toward clonal mass multiplication of plants for pharmaceutical application (Hussain *et al*., 2013). PGRs play indispensable roles in plant cell differentiation and morphogenesis and have thus been used for plant tissue culture, especially for micropropagation of Asteraceae, such as *Stevia rebaudiana Bertoni* (Chotikadachanarong & Dheeranupattana, 2013) *Gerbera jamesonii Bolus* (Minerva & Kumar, 2013) and *Globe artichoke* (Ancora *et al.*, 1981). In this study, we established an effective protocol for micropropagation of *S. marianum* using a combination of PGRs.

Table 4. Effect of different concentrations of IBA or NAA on rooting response of *S. marianum*.

Media types	Auxins (mg/l)		Rooting percentage (%)	No. of roots per shoot	Mean length of root (cm)
	NAA (mg/l)	IBA (mg/l)			
$^1/_2$ MS	0.5	0	83.3 ± 8.8	4.5 ± 0.99^{d}	2.09 ± 0.24^{c}
$^1/_2$MS	1.0	0	88.9 ± 5.1	5.2 ± 1.08^{e}	2.60 ± 0.26^{e}
$^1/_2$MS	0	0.5	82.3 ± 6.9	5.7 ± 1.08^{f}	2.60 ± 0.29^{e}
$^1/_2$ MS	0	1.0	94.5 ± 3.9	6.6 ± 1.05^{g}	3.20 ± 0.26^{g}
$^1/_2$MS	0	0	75.6 ± 5.1	3.5 ± 0.86^{b}	1.60 ± 0.27^{b}
MS	0.5	0	81.1 ± 3.8	3.9 ± 0.96^{c}	2.03 ± 0.35^{c}
MS	1.0	0	86.7 ± 5.1	4.2 ± 1.04^{c,d}	2.50 ± 0.54^{d,e}
MS	0	0.5	80.0 ± 6.7	5.1 ± 0.90^{e}	2.41 ± 0.51^{d}
MS	0	1.0	89.9 ± 5.7	5.6 ± 0.97^{f}	2.90 ± 0.26^{f}
MS	0	0	72.3 ± 3.9	3.1 ± 1.06^{a}	1.30 ± 0.26^{a}

Mean values within a column followed by the same letter are not significantly different by Duncan's multiple range test at $p<0.05$. Culture period 4 weeks. Values correspond to means ± SE.

Table 5. List of primers, their sequences, and banding pattern of both micropropagated and field-grown parent plants of *S. marianum*.

No.	Primer name	Primer sequence (5′–3′)	Total bands
1.	S1	5′GTTTCGCTCC3′	5
2.	S3	5′CATCCCCCTG3′	6
3.	S4	5′GGACTGGAGT3′	4
4.	S5	5′TGCGCCCTTC3′	3
5.	S6	5′TGCTCTGCCC3′	2
6.	S7	5′GGTGACGCAG3′	4
7.	S10	5′CTGCTGGGAC3′	2
8.	S13	5′TTCCCCCGCT3′	1
9.	S19	5′ACCCCCGAAG3′	3
10.	S102	5′TCGGACGTGA3′	2
11.	S103	5′AGACGTCCCC3′	4
12.	S105	5′AGTCGTCCCC3′	4
13.	S106	5′ACGCATCGCA3′	5
14.	S107	5′CTGCATCGTG3′	5
15.	S109	5′TGTAGCTGGG3′	3
16.	S110	5′CCTACGTCAG3′	3
17.	S111	5′CTTCCGCAGT3′	9
18.	S117	5′CACTCTCCTC3′	5
19.	S118	5′GAATCGGCCA3′	1
20.	S119	5′CTGACCAGCC3′	4

The callus was first induced from leaf of wild-grown milk thistle on MS medium containing different concentration combinations of BA and NAA. The type and the concentration of hormone led to a significant influence on the emergence of calluses (Fig. 1a; Table 1). BA and NAA on their own produced low rate of callus induction. The calluses were sad-colored and exhibited poor growth. In addition, the occurrence of callus browning was facilitated. However, the combination of BA and NAA promoted callus formation from leafs and the color grew gradually greener. At the same concentration of BA, the callus induction rate increased with increasing concentration of NAA, with concomitant color changed from yellowish-white to yellowish-green. Additionally, the calluses gradually acquired a tighter texture and kept their growth rate well. Meanwhile, when the concentration of NAA was the same, the callus induction rate showed a rising trend and then decreased with the adding of BA, and reached the maximum decrease at 1.5 mg l^{-1}. Previously, Liu and Cai (1990) induced callus from hypocotyl of *S. marianum* on MS supplemented with 0.8 mg l^{-1} NAA, 0.5 mg l^{-1} BA and 200 mg l^{-1} casein hydrolysate. Additionally, Abbasi *et al*., (2010) obtained the highest frequency (88%) of callus induction from wild-grown *S. marianum* on MS medium with 5.0 mg l^{-1} BA. Furthermore, Cimino *et al*. (2006) induced callus from cotyledons of *S. marianum* after incubation on B5 medium solidified with 2.5 g l^{-1} phytagel and supplemented with 0.05 mg l^{-1} BA and 0.5mg l^{-1} 2,4-dicholorophenoxyacetic acid (2,4-D). Here, the highest frequency (98.9%) of callus induction was achieved on MS basal medium containing 1.5 mg l^{-1} BA and 2.0 mg l^{-1} NAA. This represents a more efficient combinations of PGRs for callus formation from leaf of *S. marianum*.

GA_3 and BA have both a significant promoting activity in shoot organogenesis from callus, but the combination of GA_3 and NAA generated a higher frequency of shoot organogenesis than BA and NAA (Table 2). This result is consistent with the Abbasi's finding (2010) indicating that the combination of GA_3 and NAA produced the best shoot from a callus of *S. Marianum*. Here, the result observed when the regenerated shoot was transferred to shoot multiplication medium (Table 3) indicated that the combinations of BA and NAA to MS medium for shoot multiplication of *S. marianum In vitro* were very effective. In fact, the longest average shoot length (3.1 cm) and the largest average multiplication rate (6.6) were observed on medium with 1.0mg l^{-1} BA and 0.3 mg l^{-1} NAA (Table 3). Interestingly, other researchers have also accomplished shoot regeneration on MS medium containing a combination of BA and NAA for *chrysanthemum* (Naing *et al.*, 2015) , *Lallemantia* (Ozdemir *et al.*, 2014) , and *Yam* (Dessalegn *et al.*, 2015).

BA and NAA are widely applied to rooting of the culture of plants *In vitro*. Similar results have been well documented in several other medicinal plants, including *Gerbera jamesonii Bolus* (Minerva & Kumar, 2013), *Melocanna baccifera* (Waikhom & Louis, 2014) , *Ajuga bracteosa* (Kaul *et al.*, 2013) , *Dracaena sanderiana* (Aslam *et al.*, 2013).

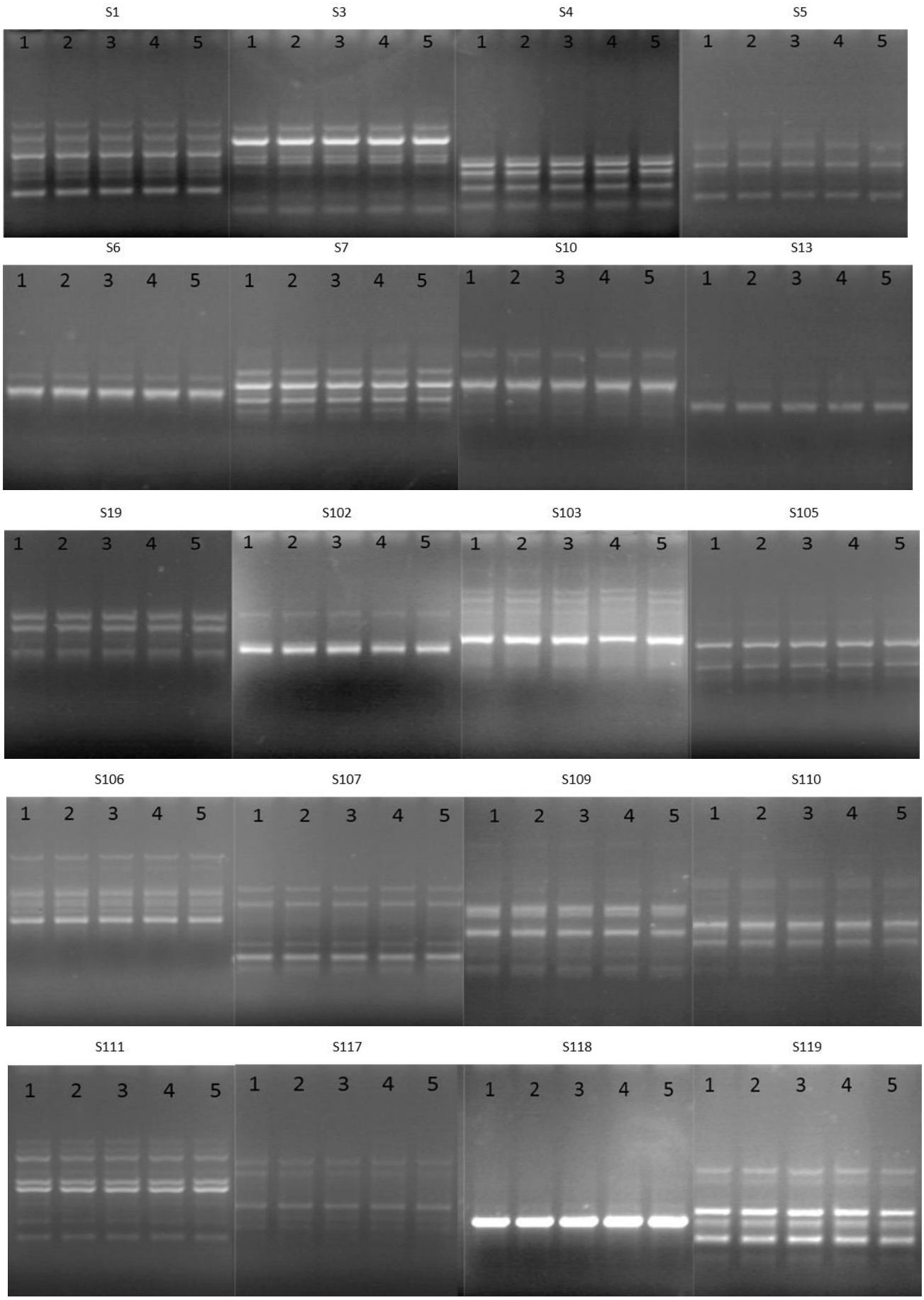

Fig. 2. Establishment of genetic fidelity between elite parent and micropropagated plants *lane1* represents parent plant; *lane2-5* represent randomly selected micropropagated plants of *Silybun .marianum.*

In this study, the MS medium containing IBA or NAA had a positive effect to some extent on rooting. The medium with IBA alone produced higher rate of rooting induction than NAA and the rooting of *S. Marianum* was more effective on $^1/_2$ MS medium. However, Abbasi (2010) found the highest frequency (73.3 ± 5.4%) of rooting for *S. Marianum* on MS medium with a mean of 4.2 ± 0.34 roots per shoot. On the other hand, here, the $^1/_2$MS medium with 1.0 mg l^{-1} IBA produced the maximum numbers of roots (6.6) and a 94.5% frequency of rooting, with a root which was stronger (Fig. 1f; Table 4).

The presence of somaclonal variation among the subclones of a single parental line during *In vitro* culture is a common occurrence observed in other plants. This is regarded as a major issue while undertaking the large-scale propagation of elite clones. The establishment of the genetic fidelity among the micropropagated plants of milk thistle employing RAPD technology was achieved for the first time here. The amplification bands were monomorphic indicating no variation between the parent plant and the micropropagated plants (Table 5). In addition, consistent with similar findings in *Desmodium gangeticum* (Cheruvathur *et al.*, 2013) , *Centaurea ultreiae* (Mallón *et al.*, 2010) and *Gerbera jamesonii Bolus* (Bhatia *et al.*, 2011), this study also found absence of genetic variability among the micropropagated plants and donor parent plants.

Conclusion

A simpler and more effective plant regeneration protocol of *S. marianum* using *In vitro* culture has been established, which provides a promising method for the large-scale *In vitro* propagation of *S. marianum*. In addition, this is the first time that the genetic fidelity in micropropagated plants of *S. marianum* was assessed by employing RAPD technology. The RAPD analysis suggested that the micropropagated plants were genetically the same as the elite parent plants. This procedure might be useful for rapid multiplication of *S. marianum* to meet the increasing demand for silymarin.

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