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Effect of elicitation on picrotin and picrotoxinin production from *in vitro* products of *Picrorhiza kurrooa*

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Picrorhiza kurrooa **Royel ex. Benth. is an important medicinal plant of Himalayan region and a good source of iridoid glycosides. Picrotin and picrotoxinin are compounds produced by *P. kurrooa* which are widely used in treatment of hepatic diseases. Elicitation is one of the best effective methods which enhance secondary metabolite production in plants. In the present study effect of elicitors for example, Methyl jasmonate (100 µM) and yeast extract (0.5 mg/ml) on the production of picrotin and picrotoxinin from *in-vitro* products of *P. kurrooa* were studied. Yeast extract (0.5 mg/ml) was found more efficient than the methyl jasmonate for enhancing the production of picrotin and picrotoxinin in roots of *P. kurrooa*. Higher amount of picrotin (2.47 µg/g dry wt.) and picrotoxinin (45.2 µg/g dry wt.) were recorded in *in-vitro* products in comparison to control plant. Genetically stable *in vitro* plants were used to assess the effect of elicitation. Genetic stability was detected with Random amplified polymorphic DNA (RAPD) and inter-simple sequence repeats (ISSR) markers. The percentage of polymorphic bands in the RAPD and ISSR analysis were 2.5 and 7.02%, respectively. The similarity coefficient revealed that differences between tissue culture raised plants and mother plant was not remarkable by both RAPD and ISSR analysis.**

Key words: Genetic fidelity, plant tissue culture, inter simple sequence repeats, West Himalaya.

INTRODUCTION

Picrorhiza kurrooa Royle ex Benth. (family: Scrophulariaceae; local/trade name: Kutki), an important medicinal herb endemic to alpine Himalaya (Thakur et al., 1989), is distributed between 2800-4800 m altitude. A group of active compounds of great pharmaceutical interest like picrotin, picrotoxinin, pikroside I and II are produced mainly in the roots and runners of this species. The extract of runners and roots of this plant has been used since long in several Ayurvedic preparations. Picrotin and picrotoxinin are used as Picrotoxin, an equimolar mixture of picrotoxinin ($C_{15}H_{16}O_6$) and picrotin ($C_{15}H_{18}O_7$). It acts as a non-competitive channel blocker

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Abbreviations: MS, Murashige and Skoog's medium; **RAPD,** Random Amplified Polymorphic DNA; **ISSR,** inter simple sequence repeats; **MeJA,** Methyl Jasmonate.

for the GABAA receptor chloride chan-nels (Rho et al., 1996). In modern medicine it is used in hepatic disorders, gastric troubles, pregnancy, anemia and asthma (Hussain, 1984; Kirtikarand Basu, 1984). Due to indiscriminate collection from wilds and lack of organized cultivation this plant is listed in 'endangered' category (Samant et al., 1998; Ved et al., 2003). Therefore, a method for rapid and efficient multiplication of this medicinal plant is highly desired.

Plant tissue culture is a prospective technique not only in the protection of natural plant resources, but also has potential commercial interest. The crucial part of any *in vitro* propagation system is mass and rapid production of plantlets which are phenotypically uniform and genetically similar to the mother plant, otherwise the advantage of desirable characters of elite/supreme clones cannot be achieved. Molecular markers such as RAPD and ISSR can be used advantageously for this purpose (Isabel et al., 1993; Rani et al., 1995; Rani and Raina, 2000; Agnihotri et al., 2009, Mishra et al., 2011a).

Plant-based molecules are continuously gaining wide spread acceptance due to their effective therapeutic properties. Production of picrotin and picrotoxinin from plant tissue cultures is important because intact plants produce this compound only in small amounts. The reported yield of active ingredients (picrotin and picrotoxinin) from field-grown plant of *P. kurrooa* is 0.64 and 7.49 µg/g dry weight (DW) (Mishra et al., 2011b). Several papers have been published on micro-propagation (Lal et al., 1988; Chandra et al., 2004; Chandra et al., 2006, Sood and Chauhan, 2011), synthetic seed formation (Mishra et al., 2011a), hairy root culture and regeneration from hairy roots of *P. kurrooa* (Mishra et al., 2011b; Rawat et al. 2013), however, assessment of secondary metabolites, that is, picrotin and picrotoxinin in tissue culture raised plants of *P. kurrooa* has not reported so far.

Elicitation enhances the accumulation of secondary metabolites and improves the productivity of plant tissue cultures (Zhao et al., 2005; Putalun et al., 2007; Kamonwannasit et al., 2008). It is also reported that, no specific elicitor has a general effect on different plant species and optimum concentration and type of elicitor is likely to vary from species to species (Putalun et al., 2010). A number of elicitors like jasmonates, salicylates, chitosan, yeast extract, ascorbic acid, and fungal culture filtrate (Komaraiah et al., 2002; Walker et al., 2002; Orlita et al., 2008; Karwasara et al., 2011a, b) have been investigated to enhance yield of plant-based secondary metabolites. MeJA has been used as an elicitor for enhancing the production of secondary compounds in plant cell cultures such as anthocyanin in *Melastoma malabathricum* (See et al., 2011) and in hairy roots cultures such as glycyrrhizin in *Glycyrrhiza inflata* (Wongwicha et al., 2011). Putalun et al. (2010) found the yeast extract enhance the production of plumbagin in the roots of *Drosera burmanii* by 3.5-fold when compared to the control plants. Similarly, Chandra and Chandra (2011) reported that elicitation with yeast extract increased decursin accumulation by 3-fold in the roots of *Angelica gigas*. Moreover, yeast extract significantly increased the intracellular content of both scopolamine and hyoscyamine in roots of *Brugmansia candida* (Pitta-Alvarez et al., 2000).

In view of this, *in vitro P. kurrooa* cultures looked favourable for the production of secondary metabolites. The present finding is the first report on the effect of elicitors for the enhancement of picrotin and picrotoxinin production from genetically stable tissue culture raised plants of *P. kurrooa*.

MATERIALS AND METHODS

Plant material

Runners of *P. kurrooa* Royle ex Benth. (common name: Kutki) were collected from Pindari area (30° 6' to 39° 15' N and 70° 55' to 80° 5' E, 3400 m amsl, District Bageshwar, Uttarakhand) in Kumaun Himalaya, India. These were brought to the laboratory and used to develop *in vitro* cultures of *P. kurrooa* as per published method (Chandra et al., 2004, 2006). *In vitro* proliferating shoot cultures of *P. kurrooa* were used for genetic fidelity analysis and elicitation.

Chemicals

All the chemicals used in tissue culture were purchased from Hi-media. All HPLC grade chemicals, MeJA and yeast extract were purchased from Sigma (Sigma Chemical Company, St. Louis, USA). Chemicals used in genetic fidelity analysis were purchased from Genei (M/s Bangalore Genei, India), whereas RAPD and ISSR primers were purchased from Operon Technologies Inc. (Alameda, California, USA) and University of British Columbia, Biotechnology Laboratory, Vancouver, Canada, respectively.

Genetic stability analysis

DNA was isolated from field grown mother plant (MP) and tissue culture raised plants (T1, T2 and T3) randomly taken from three batches.

DNA isolation, RAPD and ISSR fingerprinting

DNA was extracted using N-cetyl-N,N,N-trimethylammonium bromide (CTAB) as described by Doyle and Doyle (1987) with modifications. In brief, fresh leaf material (200 mg) was washed and ground in liquid nitrogen. Then 10 ml of preheated extraction buffer [2% CTAB (w/v), 0.2% β-mercaptoethanol (v/v), 100 mM Tris-HCl (pH 8.0), 20 mM ethylene diamine tetraacetic acid (EDTA) and 1.4 mM NaCl] were added to the powdered material. After incubating the homogenate for 1 h (at 65°C) an equal volume of chloroform : isoamyl alcohol (24:1) was added and centrifuged at 10000 rpm for 20 min. DNA was precipitated with 1/10 volume of 3 M sodium acetate and an equal volume of isopropanol followed by centrifugation at 10000 rpm for 10 min. The DNA pellet was washed with 70% ethanol, air dried and then resuspended in 200-300 µL Tris EDTA (TE; 1 mM). Quantification was performed by visualizing

under UV light, after electrophoresis on 0.8% agarose gel stained with ethidium bromide. This resuspended DNA was then diluted in sterile distilled water to 5 ng μl^{-1} concentration for use in amplification reactions.

A total of 31 random decamer oligonucleotides purchased from was used as single primers for the amplification of RAPD fragments. Polymerase chain reactions (PCRs) were carried out in a final volume of 25 µL containing 20 ng template DNA, 200 µM each deoxynucleotide triphosphate, 20 ng of decanucleotide primers, 1.5 mM MgCl2, 10 mM Tris-HCl, 50 mM KCl, 0.1% Triton X-100 and 0.5 U Taq DNA polymerase. Amplification was achieved in a Thermocycler (Biometra; Germany) programmed for a preliminary 5 min denaturation step at 94°C, followed by 40 cycles of denaturation at 94°C for 30 s, annealing at 36°C for 1 min and extension at 72°C for 1 min, finally at 72°C for 10 min.

Thirty-three ISSR primers (16-17 mer) randomly selected from primer set 9 were used for PCR amplification. All the amplification conditions were same as described above in RAPD section except annealing temperature which was 52°C for ISSR analysis.

Amplification products were separated by electrophoresis on 1.5% agarose gels run in 1X TAE (Tris Acetate EDTA) buffer, stained with ethidium bromide and visualized under UV light. Gel photographs were scanned through Gel Doc System (Alpha ImagerTM IS-2200, San Leandro, CA, USA). PCRs were repeated at least twice to establish reproducibility of results.

Data analysis

Each polymorphic band was considered as a binary character and was scored 1 (presence) or 0 (absence) for each sample and assembled in a data matrix. Only intensely stained, unambiguous, and reproducible bands were scored for analysis. Similarity index was estimated using Dice coefficient of similarity (Nei and Li, 1979). Cluster analyses was carried out on similarity estimates using the unweighted pair-group method with arithmetic average (UPGMA) using Gene Profiler 1-D Phylogenetic analysis and Data basing Software.

Elicitor treatment: Stock preparation and addition of elicitors

Methyl Jasmonate

Methyl Jasmonate (MeJA), a signaling intermediate, was dissolved in 95% ethanol, filter sterilized and tested at a final concentrations of 50, 100, 150, 200 µM.

Yeast extract

Yeast extract was prepared as an aqueous stock (10 mg/mL) having pH 5.7, filter sterilized and tested at 0.5, 1.0, 1.5, 2.0 mg/l concentrations.

Four weeks old *in vitro* established plants were used to check the effect of elicitation on secondary metabolite production. Plantlets were maintained in MS (Murashige and Skoog, 1962) liquid medium at 25 ± 1°C under 16 h light/day with agitation (80 rpm). Elicitors were added to 28-day-old well rooted plant cultures, which were harvested after elicitor treatment at 5 and 10 days. Each experiment was done in triplicate. Untreated tissue culture raised plants of the same age were used as control.

Extraction of active ingredients

In-vitro raised plants were used to extract picrotin and picrotoxinin. The fresh weight and dry weight after lyophilization were determined. The powdered samples (0.5 g DW) were extracted with 100 ml of 70% ethanol in a soxhlet apparatus, and then dried crude extract was dissolved in 10 ml of water:methanol:isopropanol: acetonitrile (60:30:5:5) and quantification by HPLC was done using standards (Purohit et al., 2008; Mishra et al., 2011b). A standard curve was prepared by 10, 20, 30, 40 and 50 mg/L solutions of picrotin and picrotoxin standard (Sigma Chemical Company, St. Louis, USA).

HPLC analysis

Purified samples (20 µL) were analyzed in a HPLC system (LaChrom, L-7100, Merck Hitachi, Japan; Column RP-18e; 5 µm; Purosphare Star, Merck). Samples were eluted in isocratic mode with water:methanol:isopropanol:acetonitrile (60:30:5:5) at a flow rate of 0.5 ml/min. Detection was done at 220 nm using UV detector L 7400. The calculations of picrotin and picrotoxinin were carried out on peak area basis using standard curves made with reference standard compound (Mishra et al. 2011b).

Statistical analysis

Results were reported as the mean ± standard deviation (SD) and analyzed by the least significant difference (LSD) test. Differences with $P<0.05$ was considered as significant.

RESULTS AND DISCUSSION

Elicitor treatment

In the present work attempts were made for the improvement in picrotin and picrotoxinin production by means of elicitor treatment to the genetically stable *in vitro* culture of *P. kurrooa* (Figures 2 and 3). MeJA and yeast extract were tested at varying concentrations.

Figure 2 shows effect of various concentration of MeJA (50, 100, 150 and 200 µM) on picrotin (Figure 2A) and picrotoxinin (Figure 2B) production. A direct correlation between incubation time (5 or 10 days) and the yield of picrotin was observed. Significant increase was observed in picrotin production after 10 days treatment of MeJA. MeJA at 50 µM gave the highest concentration of picrotin (1.56 ± 0.2 µg/g dry wt) which was 2.26-fold higher than the control plants (0.69 µg/g dry wt). In case of picrotoxinin, no significant difference was observed in cultures treated with MeJA at day 5. Maximum production (38.4 ± 2.2 µg/g dry wt) was recorded with 50 µM MeJA whereas; minimum production (30.4 ± 2.4 µg/g dry wt) was recorded with 200 µM concentration of MeJA at day 10 (Figure 2B). The results showed that various concentrations of MeJA (50-200 µM) have different eliciting influences. Similar results have been reported by Loc et al. (2014).

It has already been reported that exogenous application of MeJA to the plant cell culture or intact plant is known to stimulate biosynthesis of secondary metabolites (Mueller et al., 1993; Fang et al., 1999). Jasmonates are known as signalling intermediates in the

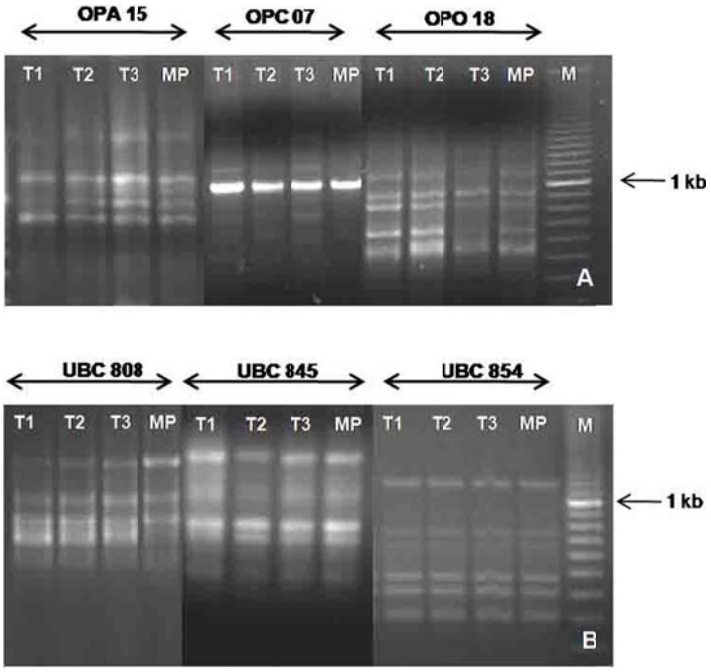

Figure 1. Assessment of genetic fidelity of tissue culture products with their parent *Picrorhiza kurrooa* A. RAPD amplification profiles with primer OPA 15 OPC 07 and OPO 18; *M* is molecular weight marker (3kb); T1-T3 tissue culture raised plants; MP is mother plant B. ISSR amplification profiles with primer UBC-808, UBC 845 and UBC-854; *M* is molecular weight marker (3kb); T1-T3 tissue culture raised plants; MP is mother plant.

wound and/or elicitor-activated expression of plant defence genes in parsley (*Petroselinum crispum*) cell cultures and transgenic tobacco (*Nicotiana tabacum*) plants (Ellard-Ivey and Douglas, 1996; Robert and John, 1997). It has also reported that MeJA stimulated a multi-component defence response in leaves and suspension-cultured cells of *Vitis vinifera* and enhanced production of artemisinin in *Artemisia annua* cell culture (Repka et al., 2001; Baldi and Dixit, 2008). Shohael et al. (2007) reported MeJA induced over-production of eleutherosides in somatic embryos of *Eleutherococcus senticosus* cultures in bioreactors.

Effect of yeast extract (0.5, 1.0, 1.5 and 2.0 mg/ml) was examined on picrotin and picrotoxinin production in *P. Kurrooa* (Figure 3). Significant increase was observed in picrotin production after 10 days treatment of yeast extract (Figure 3A). Maximum increment in picrotin (2.47 ± 0.2 µg/g dry wt, 3.8 fold) was recorded with 0.5 mg/ml yeast extract. Results showed that picrotin accumulation decreased at higher concentrations of yeast extract (1.0-2.0 mg/ml). Effect of yeast extract on picrotoxinin production is presented in Figure 3B. A clear response of plant was observed by yeast extract after 10 days (Figure 3B) for picrotoxinin production. Yeast extract at 0.5 mg/ml showed the highest influence on picrotoxinin production (45.2 ± 5.2 µg/g dry wt) which was 1.88 fold higher than the control plants. Minimum production of picrotoxinin was recorded with 2.0 mg/ml (32.0 ± 3.1 µg/g dry wt).

Results of the study are similar to those of Putalun et al. (2010), Goyal and Ramawat (2008), Turgut-Kara and Ari (2011) and Cai et al. (2012), who reported elicitation efficiency of yeast extract on the biosynthesis of isoflavonoids, cytochrome P450 and anthocyanin, and phenolic acid respectively. Wilczańska-Barska (2012) reported that yeast extract (50 mg/mL) increased acteoside production by 1.4-fold and flavone production by 1.7-fold after 7 and 14 days of elicitation. Among various carbon and nitrogen sources, glucose, peptone and yeast extract were found to be the most favourable for palmarumycin C^{13} production (Zhao et al., 2013). The probable reason for the effects of yeast extract on the enhancement of plant based secondary metabolites could be attributed to its complex composition and the presence of cations like Zn, Ca, and Co (Suzuki et al., 1985).

Analysis of genetic fidelity by RAPD and ISSR

Initial screening of 31 decamer RAPD primers resulted in selection of 16 oligonucleotides, which produced clear amplification products. Each primer produced a unique set of amplification products ranging in size from 0.3 to

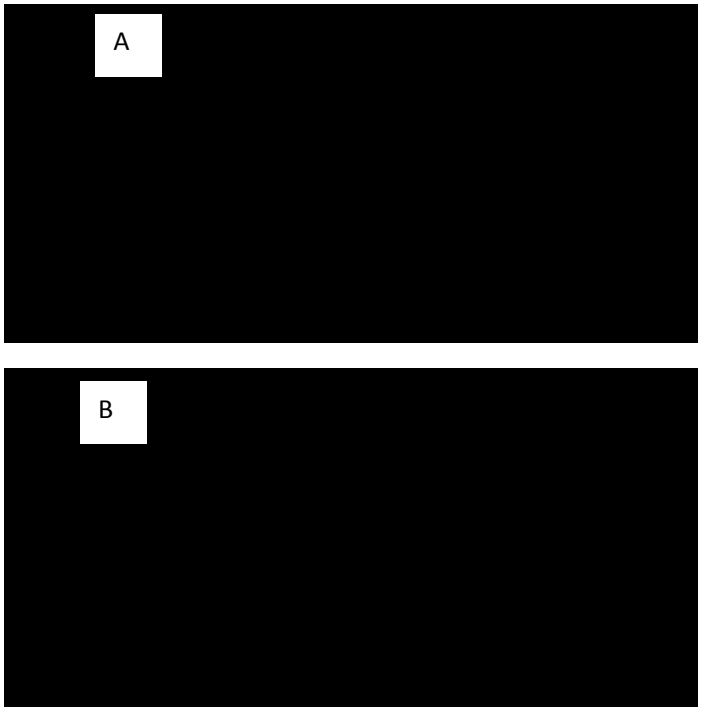

Figure 2. Effect of methyljasmonate on concentration of picrotin (A) and picrotoxinin (B) content of *P. kurrooa.*

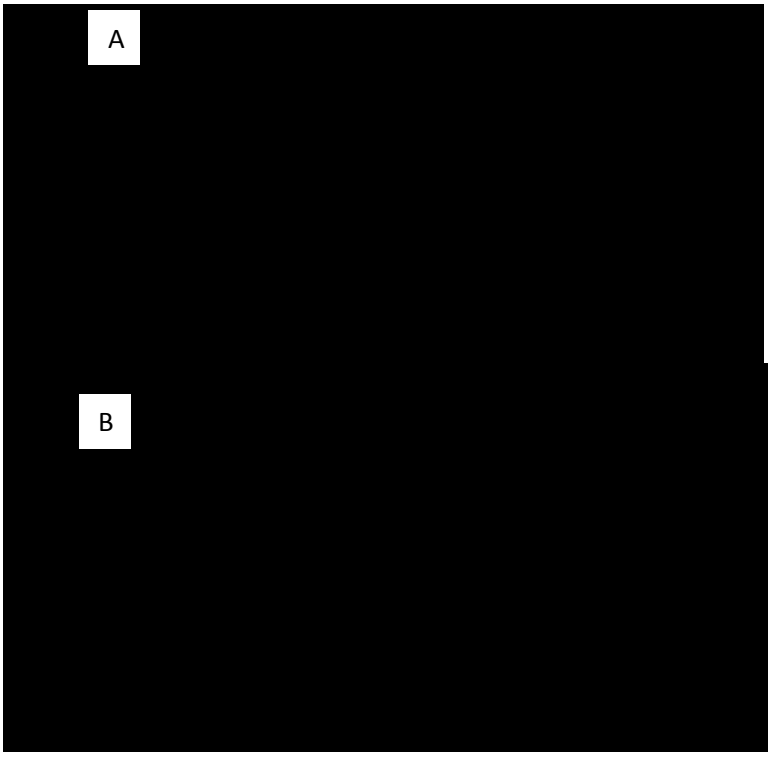

Figure 3. Effect of yeast extract on concentration of picrotin (A) and picrotoxinin (B) content of *P. kurrooa.*

Table 1. Total number and size range of amplified fragments and number of polymorphic fragments generated by 16 random primers by RAPD analysis.

Primer	Primer sequence	Total No. of amplified products	No. of polymorphic bands	Size range (kb)	% polymorphic bands
OPA-2	5' TGCCGAGCTG 3'	4	0	0.3-2.4	0
OPA-3	5'AGTCAGCCAC 3'	5	0	0.6-2.9	0
OPA-7	5'GAAACGGGTG 3'	4	0	0.5-1.6	0
OPA-10	5'GTGATCGCAG 3'	4	0	1.0-1.3	0
OPA-11	5'CAATCGCCGT 3'	7	0	0.5-2.4	0
OPA-15	5'TTCCGAACCC 3'	5	1	0.6-2.8	20
OPA-16	5'AGCCAGCGAA 3'	5	0	0.5-1.6	0
OPA-19	5'CAAACGTCGG 3'	8	0	0.5-1.8	0
OPC-2	5'GTGAGGCGTC 3'	8	0	1.0-1.3	0
OPC-7	5'GTCCCGACGA 3'	1	0	0.5-2.8	0
OPC-13	5'AAGCCTCGTC 3'	8	0	0.4-1.2	0
OPC-16	5'CACACTCCAG 3'	5	1	0.5-1.8	20
OPC-18	5'TGAGTGGGTG 3'	8	0	0.4-1.9	0
OPC-19	5'GTTGCCAGCC 3'	2	0	0.3-2.4	0
OPO-15	5'TGGCGTCCTT 3'	3	0	0.5-1.8	0
OPO-18	5'CTCGCTATCC 3'	5	0	0.3-2.4	0
Total		82	2	0.3-2.9	2.5

Table 2. Total number and size range of amplified fragments and number of polymorphic fragments generated by 12 primers by ISSR analysis.

Primer	Primer sequence	Total No. of amplified products	No. of polymorphic bands	Size range (bp)	% polymorphic bands
UBC-803	(AT)8 C	2	0	0.5-2.7	0
UBC-808	(AG)8 C	4	1	0.5-2.7	25
UBC-811	(GA)8 C	4	0	0.6-2.9	0
UBC-820	(GT)8 C	5	2	0.3-2.9	40
UBC-822	(TC)8 A	8	0	0.6-2.9	0
UBC-823	(TC)8 C	4	0	0.6-1.5	0
UBC-827	(AC)8 G	6	0	0.2-2.4	0
UBC-830	(TG)8 G	5	1	0.6-2.9	20
UBC-841	(GA)8 YC	4	0	0.5-2.7	0
UBC-845	(CT)8 RG	5	0	0.6-2.9	0
UBC-849	(GT)8 YA	6	0	0.2-1.4	0
UBC-855	(AC)8 YT	4	0	0.6-2.9	0
Total		57	4	0.2-2.9	7.02

Y= C; T and R= A,G

2.9 kb. The number of bands for each primer varied from 1 (OPC 07) to 8 (OPA-19, OPC-2, OPC-13 and OPC-18; Table 1; Figure 1A). These 16 primers used in this analysis yielded 82 scorable bands with an average of 5.12 bands per primer. Of the 82 fragments scored from these primers 80 were monomorphic and 2 were polymorphic (2.5%) producing similarity values that ranged from 0.97 to 1 with a mean of 0.985.

A total of 12 out of 33 ISSR primers were selected as suitable in terms of reproducibility of bands for the present study. The number of bands for each primer varied from 2 (UBC-803) to 8 (UBC-822) (Table 2; Figure 1B). Twelve primers yielded 57 scorable bands with an average of 4.75 bands per primer. Of the 57 fragments scored from these primers 53 were monomorphic and 4 were polymorphic (7.02%). Similarity values of the samples ranged from 0.96 to 1 with a mean of 0.98. A comparative account of RAPD and ISSR are presented in

Table 3. Summary of RAPD and ISSR amplified products from samples of *P. kurrooa.*

Description	RAPD	ISSR
Total bands scored	82	57
Number of monomorphic bands	80	53
Number of polymorphic bands	2	4
Percentage of polymorphism	2.5	7.02
Number of primers used	16	12
Average polymorphism per primer	0.12	0.33
Average number of fragments per primer	5.12	4.75
Size range of amplified fragments (kb)	0.3-2.9	0.2-2.9

Table 3. Results indicating genetic differences between them were not remarkable. Therefore, in the screening of tissue culture raised plants only chemical components and growth properties of the plants needed consideration. The use of two types of markers, which amplify different regions of the genome, allows better analysis of genetic stability or variability in the tissue culture raised plants.

In conclusion, this study represents the successful *in vitro* culture-based approach for the production of picrotin and picrotoxinin from *P. kurrooa*. Yeast extract at 0.5 mg/mL was the most effective treatment for the enhancement of picrotin and picrotoxinin. Our findings indicate that the application of elicitors can enhance the capacity of *P. kurrooa* cultures to produce high amount of secondary metabolites for pharmaceutical purposes. The results of the present study can be pooled with other yield enhancement strategies like precursor feeding, chemical and hormonal treatment for further enhancement in secondary metabolite production.

Conflict of Interests

The author(s) have not declared any conflict of interests.

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