Journal of Applied Research on Medicinal and Aromatic Plants 6 (2017) 80–91

Contents lists available at ScienceDirect

Journal of Applied Research on Medicinal and Aromatic Plants

journal homepage: www.elsevier.com/locate/jarmap

Evaluation of genetic stability and analysis of phytomedicinal potential in micropropagated plants of *Rumex nepalensis* – A medicinally important source of pharmaceutical biomolecules

Paromik Bhattacharyya[a,c,*], Suman Kumaria[a], Biswajit Bose[a], Prasenjit Paul[a], Pramod Tandon[b]

[a] Plant Biotechnology Laboratory, Department of Botany, North-Eastern Hill University, Shillong 793 022, India
[b] Biotech Park, Kursi Road, Lucknow 226021, Uttar Pradesh, India
[c] Research Centre for Plant Growth and Development, School of Life Sciences, University of KwaZulu-Natal, Pietermaritzburg, Private Bag X01, Scottsville 3209, South Africa

ARTICLE INFO

Article history:
Received 16 May 2016
Received in revised form 31 January 2017
Accepted 5 February 2017
Available online 21 March 2017

Keywords:
Natural antioxidants
Genetic stability
Gene targeted markers
Secondary metabolites
Medicinal plants

ABSTRACT

Rumex nepalensis is an important medicinal herb found in high altitude regions of India. It is a rich source of various secondary metabolites and has a widespread use in various herbal preparations. The regeneration efficacy of both direct shoot organogenesis (DSO) and indirect shoot organogenesis (ISO) was experimentally tested and the highest regeneration frequency for DSO and ISO pathways were 87.2% and 91.2% respectively. The best rooting frequency was recorded in half-strength MS medium supplemented with 2 mg/l NAA. The plantlets after acclimatization were phenotypically similar to that of the mother plant. The clonal stability of the regenerated plants from both DSO and ISO pathways were determined using Start Codon Targeted Polymorphism (SCoT) and Random amplified polymorphic DNA (RAPD) marker techniques. Although RAPD technique detected lesser degree of variability within the cloned plants (both DSO and ISO), SCoT marker could detect comparatively higher degree of genetic variability thereby proving its efficacy over the conventional molecular markers. Regeneration methodology, plant part and solvent system significantly influenced the levels of various secondary metabolites like phenols, flavonoids, alkaloids and tannins. Simultaneously, the antioxidant assays (DPPH and FRAP) detected the antioxidant activity varied tissue specifically and a significant higher value of antioxidant activity was observed within the ISO-derived plants. Higher multiplication rate coupled with enhanced clonal stability and secondary metabolite production recorded in the present report ensure the efficacy of the protocol designed for the micropropagation of this important medicinal herb species.

1. Introduction

Medicinal plants have formed the backbone of modern herbal medicine systems comprising of Ayurvedic, Unani, and Chinese pharmacopeias. Modern day medicinal formulations are devised from these age old medical pharmacopeias. Amongst the various plant families, Polygonaceae comprises a significant number of representatives having medicinal attributes. The Asian representatives of the family Polygonaceae have also shown medicinal potentials. Amongst them, *Rumex nepalensis* Spreng is one of the most important member because of its tremendous medicinal attributes having uses in Indian traditional systems of medicine (Siddha). Several bioactive compounds like anthroquinones, napthalenes, flavonoids and other phenolic entities have been reported from *R. nepalensis* which account for its wide spread usage in traditional pharmacopeias for the treatment of dislocated bones, headache, stomach problems, bleeding, tumor, inflammation, pain, constipation and tinea (Liang et al., 2010; Manandhar, 2002; Zhang et al., 2008).

Being such a broad-spectrum medicinal plant and having a wide array of usage, *R. nepalensis* deserves special attention. Unrestricted exploitation of the medicinally important plant resources to meet the ever-increasing demand by the pharmaceutical industries, coupled with limited cultivation and insufficient attempts for replenishment, have culminated in the marked depletion of the population of various plant species which were once found in

* Corresponding author.
E-mail addresses: Bhattacharyyap@ukzn.ac.za, paromik600@gmail.com (P. Bhattacharyya).

http://dx.doi.org/10.1016/j.jarmap.2017.02.003
2214-7861/

abundance. In response to habitat destruction of these species of medicinal importance, micropropagation systems have been developed for various species like *Nardostachys jatamansi* (Bose et al., 2016), *Ceropegia sanatapaui* (Chavan et al., 2014), *Aconitum violaceum* (Rawat et al., 2013) and *Picrorhiza kurrooa* (Mishra et al., 2011). As *R. nepalensis* is also used as edible vegetable it has been subjected to a high degree of anthropogenic pressures (Jain et al., 2011). Various *in vitro* approaches have been used extensively as an efficient protocol for the large scale commercial production of important medicinal plants. Furthermore *in vitro* propagation has played a prominent role in the growth of pharmaceutical industry over the past decades in a number of ways including varietal improvement, secondary metabolites production, plant cell biosynthetic capabilities for obtaining useful products, selection of high metabolite production cell lines and studying the plant metabolism (Bhattacharyya et al., 2015; Misawa 1994; Furmanowa et al., 1998; Nadeem et al., 1998; Verpoorte et al., 2002). A variety of biochemical compounds are found in the medicinal plants, of which polyphenols have received much attention because of its role in ageing related disorders (Brewer, 2011; Procházková et al., 2011). Various *in vitro* assays have revealed the powerful antioxidant activity of the polyphenols which have been consistently protective through scavenging diverse reactive oxygen species (ROS) like hydroxyl/peroxyl radical, hypochlorous acid, superoxide anion and peroxynitrile (Halliwell, 2008). Using the *in vitro* systems, production of these medicinally important bioactive entities can be enhanced significantly contributing to the bio enrichment of the plants and production of genotypically elite populations (Chavan et al., 2014).

But, the clonal regeneration system has the inherent problem of cryptic genetic defects. Generally, multiplication involving callus phase is considered to be most unreliable for clonal propagation while plantlets regenerated by branching of the axillary buds or direct somatic embryos is considered to be genetically most uniform (Rani and Raina, 2000; Varshney et al., 2001). Thus in order to monitor somaclonal variability within the micropropagated plants, it is necessary to monitor and assess the genetic constitution and stability of the *in vitro* raised plants. In order to assess the clonal stability, use of PCR-based DNA markers play an important role and is used as a valuable tool (Bhattacharyya et al., 2016, 2015; Bose et al., 2016; Devarumath et al., 2002). Keeping the above aspects into consideration, the present study was carried out to optimize the direct and indirect shoot organogenesis protocols in *R. nepalensis*. Using SCoT and RAPD markers the stability of the micropropagated plants was verified to establish the genetic fidelity of the regeneration pathway. Along with that secondary metabolite profiling estimating the phenol, flavonoid, tannin and alkaloid contents and antioxidant activity were also investigated in order to assess and design a sustainable conservation strategy for *R. nepalensis* which would result in not only genetically stable but also increased production potentials of various secondary metabolites of medicinal importance.

2. Materials and methods

2.1. Plant materials

Nodal stem segments were excised from a two year old mother plant (Fig. 1A–C) and were sterilized in accordance to the protocol given by Bhattacharyya et al. (2016).

2.2. Direct shoot organogenesis (DSO)

The shoots were regenerated in Murashige and Skoog (1962) medium supplemented with different plant growth regulators (PGRs) like 6-benzyl amino purine (BAP; 1–5 mg/l), kinetin (KN; 1–5 mg/l), Thidiazuron (TDZ; 0.5-2.5 mg/l), 2-isopentyl adenine (2iP; 0.5-2.5 mg/l), indole butyric acid (IBA; 0.1–1 mg/l) and solidified with 3 g/l of Clarigel (Himedia, India) both singly as well as in combinations. A control without any PGR was included in all experiments. The optimum pH of the culture medium was adjusted at 5.8 using NaOH or HCl prior to autoclaving at 121 °C for 20 min. The *in vitro* cultures were kept in a growth room at 25 ± 1 °C in a 16 h photoperiod and 8 h dark cycles with 50 μmol/m^2/s of light intensity. Various growth parameters like the regeneration frequency (%), average number of shoots and average shoot length (cm) were measured in order to monitor the effect of various PGRs on shoot proliferation. One explant (nodal/rhizomatous bud) was kept in each tube having ten replicates in each treatment.

2.3. Indirect shoot organogenesis (ISO)

Using nodal stem segments as explants, the indirect shoot organogenesis (ISO) via callus phase was initiated on MS medium supplemented with various auxin based PGRs like 2, 4-Dichlorophenoxy acetic acid (2, 4-D; 0.5–2.5 mg/l), Picloram (PR; 0.5–2.5 mg/l) and naphthalene acetic acid (NAA; 0.5–2.5 mg/l). Response frequency of explants showing signs of callus induction was recorded after 4 weeks of treatment. The growth of callus tissue was determined by measuring its fresh weight in each auxin treatments. In order to optimize the effect of PGRs on shoot regeneration, calli (approximately 250 mg) proliferated on optimum callus induction medium were further cultured in plant regeneration medium either singly with BAP or in combination with IBA or NAA at various concentrations.

2.4. Rooting and acclimatization

The regenerated shoots obtained from both pathways *i.e.* DSO and ISO were transferred to half strength MS medium supplemented different concentrations of auxins like IBA (0.5–3.0 mg/l), NAA (0.5–3.0 mg/l), Indole acetic acid (IAA; 0.5–3.0 mg/l) both singly as well as in combinations for induction of *in vitro* rooting both singly as well as in combinations. Various growth parameters namely (1) the *in vitro* rooting percentage, (2) average number of roots, and (3) average root length (cm). Fully rooted regenerated plantlets were removed from culture tubes and were thoroughly washed, removing the traces of solidifying gelling agent and were transferred to thermocol pots containing a potting mixture soil and sand in 1:1 ratio. Furthermore, the plants were acclimatized for a 2 weeks' time period at 25 ± 1° C under a 16 h time period (50 μmol/m^2/s of light intensity), watered with half strength MS medium without sucrose which were then transferred to glasshouse conditions (temp: 17 °C, humidity: 70%, 70 μmol/m^2/s light intensity). Survival rate (%) was recorded after 60 days of transfer to field conditions. All the experiments were set in a completely randomized block design and each experiment was repeated three times with 10 tubes per treatment. All the data were subjected to analysis of variance using SAS and means were compared using Duncan's multiple range test (Duncan, 1955).

2.5. Genetic stability analysis

DNA was extracted from fresh young leaves of the mother plant as well as randomly selected *in vitro* raised plants using Genaxy DNA extraction kit (10 each from DSO and ISO).

A total of 36 SCoT primers [designed by Collard and Mackill (2009)] were screened of which 12 best primers which gave optimum results were selected to check clonal stability of the *in vitro* propagated plants whereas in case of RAPD a total of 45 primers were screened out of which 7 primers which gave the best amplifi-