Industrial Crops and Products 76 (2015) 479–486

Contents lists available at ScienceDirect

Industrial Crops and Products

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

Establishment of adventitious root cultures from leaf explants of *Plumbago rosea* and enhanced plumbagin production through elicitation

P.K Silja, K. Satheeshkumar*

Biotechnology and Bioinformatics Division, Jawaharlal Nehru Tropical Botanic Garden and Research Institute, Palode, Trivandrum 695562, India

ARTICLE INFO

Article history:
Received 30 March 2015
Received in revised form 6 July 2015
Accepted 14 July 2015

Keywords:
Jasmonic acid
Sodium salicylate
Exudation
Normal root
5-hydroxy-2-methyl- 1
4-napthoquinone
Elicitor

ABSTRACT

Adventitious root cultures of medicinal plants are considered as a source of raw material for pharmaceutical industries. The roots of *Plumbago rosea* are used in various ayurvedic preparations for treating several diseases. Plumbagin is the major bioactive compound in the roots and it possesses activities against HIV and different types of cancers. Direct adventitious root initials were initiated on leaf explants of *P. rosea*, in Murashige and Skoog semisolid media supplemented with 1.5 mg L^{-1} indole-3-acetic acid (IAA) and 1 mg L^{-1} indole-3-butyric acid (IBA) and was used to establish root suspension cultures. Culture parameter optimization experiments showed that 3/4th strength MS liquid media with 30% sucrose and 2 g L^{-1} (FW) root inoculum was optimum for maximum biomass production. Without elicitation, maximum plumbagin production was obtained in media containing 0.5 mg L^{-1} IBA and 0.1 mg L^{-1} GA3. The study suggests that fast growing and high yielding adventitious roots could be established from leaf explants by optimizing the culture conditions for root induction and growth. Elicitation with different concentrations of elicitors such as jasmonic acid, yeast extract and sodium salicylate showed that 50 μM jasmonic acid for three days significantly increased plumbagin content in roots to 1.23% DW. The results reveal that elicitation is a good strategy for enhancing the plumbagin content in adventitious root cultures.

1. Introduction

Plants produce low molecular weight organic compounds, which have no direct role in their growth and development, known as secondary metabolites. These metabolites possess biological activities, which makes them suitable for drug discovery. In many plants, these bioactive compounds are synthesized and accumulated in roots. Among various groups, naphthoquinones represents one of the major and diverse groups of plant secondary metabolites with wide range of activities (Babula et al., 2006, 2009). The hydroxy-naphthoquinone, plumbagin is generally extracted from the roots of *Plumbago* species (Plumbaginaceae), which are used in Indian systems of medicine. Among the species, *Plumbago rosea* L. is reported to be the best source for harvesting plumbagin (Mallavadhani et al., 2002). Plumbagin is endowed with numerous vital properties such as anticancer, neuroprotective (Luo et al., 2010), tumor angiogenesis inhibition (Lai et al., 2012), prolongevity (Hunt et al., 2011), antibacterial, antifungal (de Paiva et al., 2003), anti-malarial (Likhitwitayawuid et al., 1998), antiviral (Min et al., 2001) and cardiotonic (Itoigawa et al., 1991) activities.

Various tissue culture techniques produces plant secondary metabolites and among them, culturing of differentiated tissues produce higher level of plant secondary metabolites since they are relatively more stable. Root culture is as an effective technique for commercial level production of important secondary metabolites that accumulates naturally in plant roots (Choi et al., 2000; Paek et al., 2009; Baque et al., 2012a). Several studies are already conducted in connection with establishment and scaling up of hairy root cultures of *P. rosea* (Gangopadhyay et al., 2011; Pillai et al., 2015). Since, hairy roots are genetically transformed they are not considered by pharmaceutical industries as proper source for plumbagin. Meanwhile, normal root cultures are ideal for scaling up of biomass production, which can serve as raw materials for pharmaceutical industries and also for compound isolation.

Adventitious roots are formed by direct organogenesis from cambium cells or indirectly from callus tissues arising due to mechanical damage. *In vitro* cultures of adventitious roots show a high rate of proliferation and active metabolism (Hahn et al., 2003; Yu et al., 2005). This article reports the findings of the investigations done to optimize the parameters for normal root culture established from leaf explants and elicitation of cultures for enhanced

* Corresponding author. Fax: +91 0 472 2864696.
E-mail address: bioproduction09@gmail.com (K. Satheeshkumar).

http://dx.doi.org/10.1016/j.indcrop.2015.07.021
0926-6690/

plumbagin production. To the best of our knowledge, this is the first report on elicitor induced plumbagin enhancement in adventitious root cultures of *P. rosea*.

2. Materials and methods

2.1. Initiation of adventitious roots

Aseptic shoot cultures of *P. rosea* was established using nodal explants collected from plants grown in JNTBGRI medicinal garden as described earlier by Satheeshkumar and Seeni (2003). Leaves from one-month-old aseptic shoots maintained in solid basal Murashige and Skoog (MS, 1962) media were used for the study. Leaf pieces of 10 × 10 mm size were placed on solid MS media with 30 g L^{-1} sucrose and 1.5 g L^{-1} Gelrite (SRL, Mumbai, India) supplemented with varying concentration and combinations of IAA (0.5–1.0 mg L^{-1}), IBA (0.1–1.5 mg L^{-1}) and NAA (0.5–1.5 mg L^{-1}) in Petri dishes for adventitious root induction. Cultures incubated at 25 ± 2 °C under white fluorescent light. For each different treatment, at least six explants were used and duplicates were kept for each set and experiment was repeated twice. The effect of conditions on initiation of roots from explants was recorded based on visual observations. Plant growth regulator (PGR) combination that produced best result was selected for further experiments.

Roots induced on leaf explants inoculated in MS media supplemented with 1.0 mg L^{-1} IBA and 0.5 mg L^{-1} IAA was used for establishing adventitious root cultures. Direct adventitious root initials (1–1.5 cm), without callus phase, emerged from leaf segments were excised after 4 weeks and inoculated to liquid media with same PGR combination and incubated on a gyratory shaker at 80 rpm. Roots were subcultured at regular intervals onto fresh liquid media with same combination. Experiments were conducted to optimize the culture parameters for enhancing the root biomass and plumbagin production in these established adventitious root cultures.

2.2. Optimization of culture parameters

Influence of different parameters such as type of media, media salt strength, inoculum density, and sucrose concentration on root biomass increase and plumbagin production were studied. Roots segments were inoculated to different types of full salt strength nutrient media such as MS, Woody Plant Medium (WPM), and Gamborg's B5, supplemented with 1.0 mg L^{-1} IBA and 0.5 mg L^{-1} IAA, to find out suitable media for root growth. Influence of MS media with same hormonal combination and varying salt strength ¼x, ½x, ¾x, 1x and 2x on biomass growth was studied. Roots segments were inoculated in varying fresh weights such as 1, 2, 3, 4 and 5 g L^{-1} onto 3/4x MS liquid medium supplemented with 1.0 mg L^{-1} IBA and 0.5 mg L^{-1} IAA to identify the optimum inoculum to attain maximum biomass production. Influence of varying concentrations of sucrose (10–50 g L^{-1}) on biomass and production of plumbagin was studied in MS liquid media supplemented with 1.0 mg L^{-1} IBA and 0.5 mg L^{-1} IAA.

2.3. Optimization of plant growth regulators for plumbagin production

PGRs are considered as potent stimulators for root development, growth and secondary metabolite production under *in vitro* conditions and optimization of PGR type and concentrations are essential for attaining high productivity. Roots (2 g L^{-1}, FW) were inoculated to 3/4x MS liquid media (50 mL) supplemented with 30 g L^{-1} sucrose and varying concentration and combinations of IAA (0.1–1.5 mg L^{-1}), IBA(0.5–2.5 mg L^{-1}), BAP (0.1–1.0 mg L^{-1}), GA3(0.1–1.0 mg L^{-1}) and NAA(1.0–2.5 mg L^{-1}) to study their influence on root fresh weight increase and plumbagin accumulation.

2.4. Elicitor treatment for plumbagin production

Since the effect of various elicitors on enhancement of plumbagin content in adventitious root cultures of *P. rosea* are not yet reported, an experiment was conducted to explore the influence of elicitor treatment on plumbagin production. JA stock concentration of 100 mg mL^{-1} was prepared in DMSO and diluted with distilled water to obtain a working concentration of 100 mM. YE and SS solution was prepared as an aqueous stock (10 mg mL^{-1}) and adjusted pH to 5.7 and autoclaved before use.

Roots (2 g $L^{-1,}$ FW) from log phase cultures (14 days old) were inoculated to fresh MS 3/4x strength liquid medium supplemented with 1 mg L^{-1} IAA and 0.5 mg L^{-1} IBA. Inoculated flasks were incubated in a gyratory shaker at uniform conditions for 16 days. Elicitors were added aseptically to individual flasks on 16th day of cultivation. JA was added to cultures to give final concentrations of 50, 100, and 200 μM and in another two different set of cultures, sterile YE and SS was to get final concentrations of 50, 100 and 200 mg L^{-1}. Control flasks were kept without adding elicitors and the experiments were performed in triplicate. After addition of elicitors, the cultures were incubated on a gyratory shaker at uniform conditions. Cultures were harvested at 3rd and 6th days after addition of elicitors to monitor their influence on biomass growth and plumbagin production. The harvested roots were rinsed with distilled water, blotted on a tissue paper and fresh weights were determined. Dry weights of the roots were recorded after two days of drying at 40 °C in a hot air oven. The fresh root growth index was measured as

$$\text{Growth index (GI)} = \frac{\text{Final FW} - \text{Initial FW}}{\text{Initial FW}}$$

Plumbagin exuded to the media on addition of elicitors, was separated using methanol and chloroform. Plumbagin was extracted by over overnight static extraction of the dried, ground roots with chloroform at room temperature. HPLC estimation of plumbagin was done according to Crouch et al. (1990), on a Gilson 321 Liquid chromatographic system with a Kromasil 100C (250 × 4.6 mm) C-18 column using methanol-acetic acid (80:20) buffered with trimethyl amine (pH 3.5). The flow rate was 1.0 mL min^{-1} and identification of the compound was based on co-chromatography with authentic plumbagin and their retention time at 254 nm (UV/VIS-156).

2.5. Statistical analysis

Twelve explants per each combination were used in root initiation experiment and experiments with root suspension cultures were conducted twice in triplicate. Data was recorded after four weeks of incubation, if not mentioned otherwise. Statistical analysis was carried out using analysis of variance (ANOVA) comparing the treatments and using Duncan's multiple range test at the 5% probability and analyzed using SPSS for Windows, version 20.

3. Results and discussion

3.1. Initiation of adventitious roots

Adventitious roots were initiated on leaf explants in media with different combinations of plant growth regulators (PGR) after a period of 10–15 days. The time for root induction varied with concentrations and combinations of PGRs present. Root induction was observed in all media supplemented with combinations of IAA with IBA (Fig. 1). Media with high IAA concentration together with