Plant Physiology and Biochemistry 125 (2018) 106–115

Contents lists available at ScienceDirect

Plant Physiology and Biochemistry

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

Research article

Piriformospora indica cell wall extract as the best elicitor for asiaticoside production in *Centella asiatica* (L.) Urban, evidenced by morphological, physiological and molecular analyses

S. Jisha[a], P.R. Gouri[a], K.N. Anith[b], K.K. Sabu[a,*]

[a] Division of Biotechnology and Bioinformatics, Jawaharlal Nehru Tropical Botanic Garden and Research Institute, Thiruvananthapuram, 695562, Kerala, India
[b] Division of Microbiology, Kerala Agricultural University, Thiruvananthapuram, 695 522, Kerala, India

ARTICLE INFO

Keywords:
Piriformospora indica cell wall extract
Centella asiatica
HPLC
β amyrin synthase

ABSTRACT

Vascular plants synthesise a multitude of organic molecules or phytochemicals, referred to as "secondary metabolites". These molecules are involved in a variety of roles in the life span of plants, ranging from structural ones to protection. *Centella asiatica* (L.) Urban has probably been used since prehistoric times and has been reported to have been used for various medicinal and cosmetic purposes. The plant contains several active constituents, of which the most important is asiaticoside, a triterpenoid. Asiaticoside content in *C. asiatica* can be enhanced by the use of biotic elicitors like *Piriformospora indica. P. indica* has been used as a model to study the mechanisms and evolution of mutualistic symbiosis. *P. indica* is similar to Arbuscular Mycorrhizal (AM) fungi in terms of plant growth promotional effects. The autoclaved fraction from *P. indica* (PiCWE) was found to be the most active fraction in promoting the plant biomass and asiaticoside content. To date, there are no reports on the potential role of PiCWE in enhancement of asiaticoside over the control and *P. indica* colonized plants, which was evidenced by the differential expression of key genes involved and final asiaticoside content along with the determination of phytohormones. Moreover, differential expression of selected miRNAs in PiCWE - *C. asiatica* root interactions over the control and *P. indica* treated *C. asiatica* leaf samples was also scrutinized. The important consequence of induction with PiCWE was the significant enhancement of asiaticoside in the PiCWE induced plants in comparison with the asiaticoside content in control and *P. indica-C. asiatica* interaction. In addition, the role of miRNAs in *C. asiatica* – PiCWE would enable more in-depth studies for deciphering the molecular and physiological mechanisms of the association and regulation of PiCWE - *C. asiatica* interactions.

1. Introduction

Elicitation is the process of inducing or enhancing synthesis of secondary metabolites by the plants to ensure their survival, persistence and competitiveness (Gorelick and Bernstein, 2014, 2017). Plant cells *in vitro* show physiological and morphological responses to microbial, physical or chemical factors which are the elicitors. Various elicitors have been reported to control metabolic flux between the steroid and the triterpene pathways, probably by acting at the level of cyclases. Efforts to elicit the biosynthesis of centellosides mainly focus on usingMethyl Jjasmonate (MJ), thidiazuron (Kim et al., 2004), and a permeabilization and feeding strategy by treatment with DMSO alone or in combination with β Amyrin (Hernandez-Vazquez et al., 2010) in cell cultures, roots and whole plants of *Centella asiatica*. Efforts to improve the triterpenoid content of *C. asiatica* in *in vitro* shoot cultures by elicitation with exogenously supplied MJ resulted in a significant enhancement of the triterpenoid content at the expense of plant growth and decreased free sterol content (Mangas et al., 2006). However, eliciting transformed hairy root cultures of *C. asiatica* with MJ enhanced asiaticoside production as well as root biomass (Kim et al., 2007). The raise in secondary metabolite production effected by endosymbiosis is apparently due to elicitation of plant defense in response to fungal elicitors like lipopolysaccharides and glycoproteins formed by the action of plant derived hydrolases secreted in response to endophyte colonization (Gao et al., 2010; Netzker et al., 2015). Symbiotic nitrogen fixation is a longer term relationship often involving a special structure to house a microbial partner. Nitrogen fixing symbiotic association involves a nitrogen fixing symbiotic organism - the microsymbiont like

Abbreviations: MJ, Methyl Jasmonate; DMSO, Methyl Sulfinyl Methane; AM, Arbuscular Mycorrhiza; DSM, Deutsche Sammlung fur Mikroorganismen; PiCWE, *P. indica* Cell Wall Extract; SQS, Squalene Synthase; β AS, β Amyrin Synthase; CYS, Cycloartenol Synthase; miRNA, micro RNA; IAA, Indole Acetic Acid; t-ZR, trans-Zeatin Riboside; PDA, Potato Dextrose Agar; PDB, Potato Dextrose Broth; BHT, Butylated HydroxylToluene

* Corresponding author.
E-mail address: sabu@jntbgri.res.in (K.K. Sabu).

https://doi.org/10.1016/j.plaphy.2018.01.021
Received 18 December 2017; Received in revised form 16 January 2018; Accepted 22 January 2018
Available online 07 February 2018
0981-9428/

Rhizobium, Klebsiella, Nostoc or *Frankia* and a eukaryotic photosynthetic host like leguminous or nonleguminous plant, waterfern or liverwort (Graham and Eissenstat, 1998). *Piriformospora indica* is an endophytic axenically cultivable fungus with Arbuscular Mycorrhizal (AM) fungi like growth promotional effects (Monreal and Dalpe, 2013).

Piriformospora indica, discovered in the Indian Thar desert in 1997 (Varma et al., 2001), is related to the *Sebacinales* [ordo nov.] (form genus *Rhizoctonia*; *Hymenomycetes, Basidiomycota*) (Weiss et al., 2004). *P. indica* was originally isolated in association with a spore of *Glomus mosseae* from the rhizosphere of two shrubs (Krishnaveni et al., 2014) and was named as DSM 11827 (deposited at the Deutsche Sammlung fur Mikroorganismen und Zellkulturen, Braunschweig, Germany; Varma, 1998). This fungus has been used as a model to study the mechanisms and evolution of mutualistic symbiosis. Like AM fungi, *P. indica* is able to transfer growth-promoting activity to its host plants but it possesses a broader host range among mono- and dicotyledonous plants (Varma et al., 1999; Pham et al., 2004; Barazani et al., 2005; Sherameti et al., 2008; Deshmukh et al., 2006). In spring barley, *P. indica* colonization enhanced plant biomass which was accompanied by grain yield increases of up to 11%. *P. indica* stimulates adventitious root formation in ornamental cuttings (Pham et al., 2004), while enhanced salt tolerance has been observed in barley (Waller et al., 2005). The endophytic fungus *P. indica* colonizes the plant roots and promotes their performance, biomass and seed production as well as resistance against biotic and abiotic stress (Vahabi et al., 2013; Oelmuller et al., 2009). Vadassery et al. (2009) reported the isolation of a fraction from the cell wall of *P. indica,* PiCWE.

Centella asiatica (L.) Urban is a clonally propagated tropical medicinal plant and it contains several active constituents, of which the most important are the triterpenoid saponins, including asiaticoside, madecassosic acid and asiatic acid (Aziz et al., 2007). Asiaticoside is the major trisaccharide triterpene (Inamdar et al., 1996; Maquart et al., 1999) since asiaticoside shows profound medicinal properties (Hausen, 1993). The present study includes a detailed analysis of the differential expression of the key genes involved in asiaticoside pathway [Squalene synthase (SQS) and β Amyrin synthase (β AS)] and a phytosterol synthesising Cycloartenol synthase (CYS) in comparison with the differential expression of selected miRNAs in *C. asiatica* - *P. indica* interactions. miRNAs, a class of short 17–24 nucleotide non-coding RNAs that direct post-transcriptional repression of messenger RNAs, increasingly have been shown to play a key role in regulating cellular physiology. The interest that miRNA acquired in such a short span of time indicates that its impact cannot be underestimated and many studies have been conducted on miRNA biogenesis and its functions (Gregory et al., 2005). Recent reports regarding the potential application of targeting miRNAs is increasing in gene therapy testing and preclinical studies are available (Almeida et al., 2011). MiRNAs are mainly involved in different plant functions like its development, signal transduction and protein degradation and regulate the expression of many important genes; a majority of these genes are transcriptional factors. Apart from its' role in growth and development, they also play a key role in plant stress responses which may be either biotic or abiotic factors. Tang et al. (2012) revealed the rapid upregulation of miRNAs in response to wounding and other stress stimuli. Generally plants respond to biotic factors by regulating the expression of genes involved in different pathways along with the major role played by miRNAs. Micro RNA (miRNA) expression profiling studies have been carried out in *C. asiatica* to study the photoprotective effect of *C. asiatica* extract against UV irradiation (An et al., 2012). The present study also explores the role of phytohormones, auxin (IAA) and cytokinins (t-ZR) in the interaction between *C. asiatica* and *P. indica* cell wall extract (PiCWE) and the hormone dependent biomass enhancement in response to *P. indica*. To date, there are no reports on the potential role of PiCWE in enhancement of asiaticoside over the control and *P. indica* colonized plants.

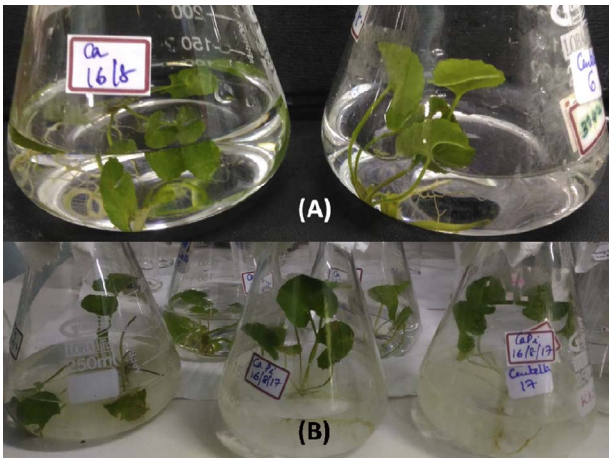

Fig. 1. *Centella asiatica* (L). Urban, Syn: *Hydrocotyle asiatica* (Hydrocotyle, Indian pennywort, Gotukola) (Fig. 1A) used in the study. *C. asiatica* was co-cultured with *P. indica* (Fig. 1B) in MS-PDB in 1:1 ratio under controlled conditions in the Plant Tissue Culture Laboratory, Jawaharlal Nehru Tropical Botanic Garden and research Institute, Kerala, India.

2. Materials and methods

2.1. Plant material

Centella asiatica (L). Urban plants from Thiruvananthapuram district of Kerala (India) were collected and the specimen was authenticated based on published identification features (Emboden and William, 1985) and are maintained in a green house of Jawaharlal Nehru Tropical botanic Garden and Research Institute, Thiruvananthapuram. Hydroponic cultures of control *C. asiatica*, *P. indica* colonized and PiCWE treated *C. asiatica* (Fig. 1) were maintained for further studies.

2.2. Initiation and maintenance of P. indica culture

Piriformospora indica cultures were provided by Dr Anith, KN, Department of Microbiology, College of Agriculture, Kerala Agricultural University, Vellayani, Thiruvananthapuram, originally gifted by Prof. Ajit Kumar Varma; School of Life Sciences, Jawaharlal Nehru University, New Delhi, India. The cultures were maintained in Potato Dextrose Agar (PDA) medium at pH 7.0 and incubated in the dark at 28 °C for a period of 10 days. Fungal hyphae (100 mg) were transferred to Potato Dextrose Broth (PDB) maintained under same growth conditions as above.

2.3. Preparation of the PiCWE

PiCWE was prepared using the protocol of Anderson-Prouty and Albersheim (1975) with modifications. Mycelia from 14-day-old liquid cultures were homogenised using mortar and pestle in 5 ml water per g of mycelia. The homogenate was filtered using miracloth (Calbiochem, Nottingham, UK). The residue was washed three times with water, once with chloroform/methanol (1:1) and finally in acetone. This preparation was air dried and the mycelial cell wall material was recovered. Elicitor fractions were prepared from mycelial cell walls by suspending 1 g of cell wall in 100 ml water and autoclaving for 20 min at 121 °C. Autoclaving releases the active fraction. The suspension was centrifuged at 14,000 rpm for 10 min, filter-sterilized using a sterile 0.45 μm Millex syringe driven filter unit (Millipore Corporation, Bedford, USA) and used for further assay. This PiCWE was used for all the molecular and biochemical studies in *C. asiatica*.