Phytochemistry Letters 17 (2016) 36–44

Contents lists available at ScienceDirect

Phytochemistry Letters

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

Short communication

UVC-priming mediated modulation of forskolin biosynthesis key genes against *Macrophomina* root rot of *Coleus forskohlii*—A tissue culture based sustainable approach

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ARTICLE INFO

Article history:
Received 10 December 2015
Received in revised form 4 June 2016
Accepted 9 June 2016
Available online 2 July 2016

Keywords:
Callus
Diterpene synthase (CfTPS)
GGPP synthase
Macrophomina phaseolina
Ultraviolet-C

ABSTRACT

Coleus forskohlii is susceptible to root rot disease that reduces yield of root specific metabolite forskolin. In this communication, we first time reported sustainable management of *Macrophomina phaseolina* root rot in *C. forskohlii* via Ultraviolet-C (UVC)-hormesis using tissue culture. Compact calli were developed using 2,4-dichlorophenoxy acetic acid (2.0 mg l^{-1}) and tolerance level of UVC exposure was optimized. Calli exhibited 88% viability, 8.4% relative growth in biomass and higher absorbance in 2,3,5-triphenyl tetrazolium chloride analysis subsequent to 10 min of UVC exposure. Pathogenicity of *M. phaseolina* was reduced in UVC-primed calli-regenerated plants compared with un-primed ones under controlled condition. Plant height, leaf number, shoot and root biomass significantly improved in UVC-primed calli-regenerated plants over un-primed ones when challenged by *M. phaseolina* in glasshouse. Significant elevation of economically important forskolin via up-regulation of key forskolin biosynthesis genes *GGPP synthase*, *CfTPS2* and *CfTPS3* highlighted the potentiality of UVC-priming to boost plant's defence mechanism and protect from drastic economic loss.

1. Introduction

Coleus forskohlii (Willd) Briq. (Lamiaceae), commonly known as Indian *Coleus*, is a perennial medicinal herb of commercial importance. It grows wild in tropical and subtropical region. The tuberous root of this plant has ethnomedicinal significance against a myriad of ailments. Forskolin, a labdane diterpene, has been identified as a principle bioactive lead/scaffold in the roots of *C. forskohlii* (Bhat et al., 1977). Forskolin has been reported to be a potent activator of adenylate cyclase, which leads to increase the level of cAMP and thereby affecting heart action, blood pressure and intraocular pressure (Engprasert et al., 2004). A semi-synthetic forskolin derivative has been clinically used in Japan for Cardiac surgery complications, heart failure and cerebral vasospasm (Toya et al., 1998; Kikura et al., 2004). Japanese also use forskolin eye drop solution for treatment of glaucoma (Wagh et al., 2012). Besides these, many other herbal/nutraceutical companies around the world are successfully employing forskolin in their marketed formulations. Hence, forskolin has gained an increasing market demand around the world. Based on the identification of putative labdane intermediates to forskolin in hairy root cultures of *C. forskohlii*, a pathway from trans-geranylgeranyl diphosphate (GGPP) to forskolin has been hypothesized by Asada et al. (2012). *GGPP synthase*, an important branch point prenyltransferase enzyme in terpenoid biosynthesis, catalyses the consecutive condensation of an allylic diphosphate with three molecules of IPP to produce Geranylgeranyl diphosphate (GGPP). *GGPP synthase* has been considered as an important regulatory target in the forskolin biosynthetic pathway (Engprasert et al., 2004). On the other hand, 13R-manoyl oxide (MO) is proposed to be the first dedicated intermediate in forskolin biosynthesis is synthesized in specialized root cork cells in *C. forskohlii* (Asada et al., 2012). Oxidation of C-11 in manoyl oxide might be a key step in the biosynthesis of forskolin. Recently, Zerbe et al. (2013) reported the sequencing and

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http://dx.doi.org/10.1016/j.phytol.2016.06.007
1874-3900/

assembly of a root transcriptome of *C. forskohlii* using 454 and Illumina sequencing technologies. Mining of the transcriptome databases resulted in the identification of a small gene family of diterpene synthase candidates (*CfTPSs*) in *C. forskohlii*. Pateraki et al. (2014) recently described *CfTPS2*, which synthesizes the intermediate copal-8-ol diphosphate, in combination with *CfTPS3*/*CfTPS4* resulted in the stereospecific formation of (13R) MO from GGPP.

Despite earlier reports on successful chemical synthesis of forskolin the synthetic approach of forskolin production was not commercially promising (Engprasert et al., 2004). Therefore, the principle source of forskolin is still endured in the roots of *C. forskohlii* (Gupta, 1988). Recently farmers have started to grow it as a crop (Vishwakarma et al., 1988) in India with annual production of about 100 tons from 700 ha (Singh et al., 2011). Presently, about 40,000 acres are under *C. forskohlii* cultivation in India, Africa and South East Asia. Indiscriminate collection of *C. forskohlii* has led to rapid depletion of wild populations resulting in its listing as a vulnerable plant species to be extinct (Gupta, 1988). Moreover, the plant is susceptible to many diseases among which root rot is the most pre-dominant (Shyla, 1998; Kamalakannan et al., 2006; Khatun et al., 2011) that ultimately leads to massive loss of root metabolite forskolin. Therefore, herbal industries faced a great challenge due to lack of sustainable management of root rot disease. *Macrophomina phaseolina*, a soil inhabiting fungus, has been identified as major causative organism for root rot in *C. forskohlii* (Kamalakannan et al., 2006). Application of pesticides in medicinal plant cultivation is not a recommended process, which significantly increases pesticide residues and heavy metal/metalloids content in herb as well as in final formulation above permissible limit. Pesticide residue also causes significant environmental hazard (Nigro et al., 1998). Besides, the important economic part of *C. forskohlii* is root; drenching of root with chemicals are not logical as these may establish imbalances in the microbial community unfavourable for activities of beneficial microorganisms (Jeyarajan et al., 1991). Therefore, development disease resistant clone would be an acceptable approach and healthy plant propagation would be the choice in agrotechnology. The application of the low doses of ultraviolet light especially Ultraviolet-C (UVC) (190–280 nm)-hormesis has emerged as an alternative technology (Stevens et al., 1998) to develop host resistance and has been approved by the FDA, USA (Rhim et al., 1999). UVC irradiation acts an inducer of phytoalexin accumulation above inhibitory levels against pathogens and thereby induces resistance in a number of species (Wilson et al., 1994; Stevens et al., 1998).

The present study aimed to develop *Macrophomina* root rot resistant clones of *C. forskohlii* by priming the *in vitro* callus of *C. forskohlii* with UVC-hormesis. The tolerance level to UVC has been optimized with respect to cell viability. The *in vitro* regenerated plants were examined with respect to disease resistance, growth pattern, and forskolin yield. The potentiality of UVC priming on expression of key forskolin biosynthesis genes was assessed using semi-quantitative RT-PCR. Expression profiling of *GGPP synthase* and diterpene synthase candidate (*CfTPSs*) genes like *CfTPS2*, *CfTPS3* and *CfTPS4* was analysed. The probable mechanism of UVC priming to develop innate immunity in tissue culture raised *C. forskohlii* was evaluated in this study.

2. Experimental

2.1. Fungal culture

M. phaseolina slants were obtained from National Bureau of Agricultural Important Pests, Bangalore. Mycelial tips from whitish fungal colonies were inoculated on sterile Potato Dextrose Agar (PDA) medium (Himedia, India) in petri dishes. The petri dishes were sealed with paraffin and kept 27 °C for 3 days and stored at 4 °C. Pure culture was maintained by repeated sub-culturing in PDA and stored at 4 °C and renewed once in every month.

2.2. Plant material

C. forskohlii maintained *in vitro* (Dube et al., 2011) in plant tissue culture laboratory in Department of Agricultural Biotechnology, Ramakrishna Mission Vivekananda University, Kolkata, India was used as source material for this experiment. The culture was developed from *C. forskohlii* cuttings collected from Agri-Horticultural Society, Alipore, Kolkata, India.

2.3. Callus induction

Leaf explants were aseptically dissected from *in vitro* maintained *C. forskohlii* and used for callus induction. Single leaf explants were inoculated in sterilized semi-solid Murashige and Skoog (MS) medium (Murashige and Skoog, 1962) fortified with 3% (wv^{-1}) sucrose and 2,4-dichlorophenoxy acetic acid (2,4-D) (2.0 mg l^{-1}). Cultures were aseptically maintained for two weeks at 22 ± 2 °C and 16 h photoperiod (light intensity 40 μmol m^{-2} s^{-1} provided by cool white fluorescent tubes, Philips India). An experimental set without 2,4-D treatment was maintained in this experiment as control. After two weeks, a well grown callus (C21) was segmented into three pieces (approximately 5–6 mm^2 in each) and placed separately in test tube containing semisolid MS media fortified with 3% sucrose and 2,4-D (2.0 mg l^{-1}) and kept for another two weeks for gaining greater biomass.

2.4. UVC priming and cell viability testing using TTC

Calli were removed in Petri dishes and subjected to UVC (254 nm) exposure for 5, 10, 15 and 30 min from 10 cm distance of UVC source in a sterile UV chamber following established protocol of Ghosh and Pal (2012) in Saha Institute of Nuclear Physics, Kolkata, India. Each petriplate consisted three calli for each time and the experiment was conducted with three replicates per UVC treatment. The entire experiment was conducted three times. Calli, unexposed to UVC, were used as control throughout experiment. After exposure, all calli along with controls were transferred singly to a test tube containing solid MS medium gelled with 0.8% (wv^{-1}) Bacto agar and fortified with 1.5 mg l^{-1} N_6-bezylaminopurine (BAP) (Himedia, India) for two weeks at 22 ± 2 °C and 16 h photoperiod. After two weeks, the relative growth rate of UVC-treated callus was determined using formula RG (%) = 100 × (W2 − W1)/t (Ingestad and Lund, 1986); where RG = %fresh weight (FW) increase, W1 = initial biomass at harvest time t_1 (DW), W2 = biomass at the harvest time t_2 (FW) and $t = t_2 - t_1$, time of the growth (days).

The viability of UVC-treated callus at different exposure time points was determined by the commonly used biochemical marker 2,3,5-triphenyl tetrazolium chloride (TTC) following the protocol of Mikula et al. (2006), which is based on the reduction of tetrazolium salts to red coloured end products in viable cells. In a TTC assay, cell survival was estimated by the amount of formazan produced from the reduction of TTC due to the action of dehydrogenases in living cells or tissue (Steponkus and Lamphear, 1967). Briefly, after two weeks of radiation exposure small segments (0.2 g) from the calli irradiated with UVC at different exposure-time points were cut and used for viability test. The calli segments were washed and dissolved in sterilized water and then centrifuged at 5000 rpm for 5 min and the water was replaced with 1.5 ml of TTC solution (0.6% TTC in Tris buffer, pH 7.5). Cells were incubated for 24 h at 30 °C in the dark and then washed with