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Stress analysis and cytotoxicity in response to the biotic elicitor, *Piriformospora indica* and its' cell wall extract in *Centella asiatica* L. Urban

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ABSTRACT

Study reports prominent findings on the analysis of oxidative stress generated in *Centella asiatica*, a multifunctional medicinal plant on co-cultivation with *P. indica* and its extract, *P. indica* Cell Wall Extract. The presence of *P. indica* had protective role in alleviating stress, evidenced by lack of significant change in H_2O_2, increased total antioxidant capacity and phenolics in *P. indica* colonized and PiCWE treated plants. No variation was observed between IC50 values and the cell integrity in *P. indica* colonized *C. asiatica* appeared intact by TEM analysis. The study preludes the possible role of *P. indica* in conferring drought tolerance to *C. asiatica*.

1. Introduction

Plants are under constant interaction by various microorganisms that aim to acquire adequate amount of nutrients from them. As a result of these interactions, the outcome can be neutral, harmful (as in the case of parasitism), or beneficial (in the form of mutualistic symbiosis) to the host [41,50]. In mutualistic associations, plant-microbe interaction is considered to be well-balanced [5]. Among this, fungi play an essential role in upgrading plant growth, vigour and survival by a positive impact on the nutritional [43] status of the plant and on soil aggregation [6,51] and the microsymbiont in turn perceives its resource for nutrition *ie*. carbon provided by the plant for its physiological functions, growth and development. Biochemical, physiological and molecular characterization on plant–microbe interactions at different levels found that microbial interactions mostly lead plant responses toward stresses [12]. The enhanced generation of free radicals or reactive oxygen species (ROS) results in oxidative stress and thus antioxidant capacity of a biological system will get declined [64]. Antioxidants are free-radical scavengers that offer resistance to living organisms from damage caused by ROS. On the contrary, these microbial elicitors can be used to alter cell metabolism of many helpful plants to increase the production of economically important compounds in plants which are the major source of various distinct drugs in the pharmaceutical industry [19]. The vast majority of such elicitors in plants are produced by fungi and bacteria [31]. *Piriformospora indica*, discovered in the Indian Thar desert in 1997 [55], is related to the *Sebacinales* [ordo nov.] (form genus *Rhizoctonia*; *Hymenomycetes, Basidiomycota*) and is a known symbiotic fungus which can promote plant growth [59].

P. indica was originally isolated from the spore of *Glomus mosseae* from the rhizosphere of two shrubs namely *Prosopis juliflora* and *Ziziphus nummularia* [24] and was named as DSM 11827 [54]. The endosymbiotic fungus *P. indica* is axenically cultivable, possesses a broad host spectrum and positively affects different aspects of plant performance [56]. Qiang et al. [35] reported that *P. indica* may represent a missing link between a saprophytic fungus and an obligate biotrophic mutualist. This unique combination of attributes makes *P. indica* and its close relatives among the *Sebacinales* very interesting tools for crop improvement [13]. It grows inter- and intracellularly and forms pear shaped, auto fluorescent chlamydospores within the cortex of the colonized roots and in the rhizosphere zone, but it does not invade the endodermis and the aerial parts of the plants [43]. Further, it is shown to stimulate biomass production, early flowering, seed production and is a potential microorganism imparting biological hardening to tissue culture-raised plants [54,61]. *P. indica* Cell Wall Extract (PiCWE) is the active fraction from the liquid culture of *P. indica*. Fungal cell wall can be isolated and autoclaved to obtain the active fractions of *P. indica*. Vadassery et al [53] reported that these active constituents of the endophytic fungus *P. indica* also stimulate enhanced growth and seed production in *Arabidopsis thaliana*. This heat-stable fraction is able to

Abbreviations: PiCWE, *P. indica* Cell Wall Extract; HRP, Horse Radish Peroxidase; MTT, 3- (4, 5- dimethylthiazol-2-yl)-2, 5- diphenyltetrazolium bromide; PDA, Potato Dextrose Agar; PDB, Potato Dextrose Broth; ATCC, American Type Cell Culture; ELISA, Enzyme Linked ImmunoSorbent Assay; DMSO, Dimethyl sulfoxide; DMEM, Dulbecco's Minimum Essential Medium; FBS, Foetal Bovine Serum; TEM, Transmission Electron Microscopy

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stimulate root and shoot growth.

Centella asiatica (L). Urban is clonally propagated plant from Umbelliferae/Apiaceae family. It has a broad spectrum of medicinal properties and is traditionally used for the treatment for leprosy, varicose veins, ulcers, lupus, certain eczemas, and mental retardation [40,47]. Because of large scale and unrestricted exploitation of this natural resource coupled with limited cultivation and insufficient attempts for its replenishment the wild stock of this species has been markedly depleted and now it is listed as threatened species by the International Union for Conservation of Nature and Natural Resources (IUCN) and an endangered species [39,44]. According to the report prepared by the Export and Import Bank of India, *C. asiatica* is one of the important medicinal plants in the international market of medicinal plant trade [33,36].

The establishment of *P. indica* root colonization in *C. asiatica* coupled with enhanced production of the major triterpenoid, asiaticoside has already been reported [20]. But till date, there are no reports available regarding the oxidative stress and cytotoxicity levels in *C. asiatica* in response to *P. indica* colonization and PiCWE treatment, as evidenced by generation of H_2O_2, total phenolics, antioxidant capacity assay, *in vitro* cytotoxicity assay etc.

2. Materials and methods

2.1. Plant material

C. asiatica (L). Urban plants from Thiruvananthapuram district of Kerala were collected and the specimen was authenticated based on published identification features [11] and are maintained in the green house of Jawaharlal Nehru Tropical botanic Garden and Research Institute, Palode, Thiruvananthapuram for further studies.

2.2. Initiation and maintenance of P. indica culture

P. 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 Cell Wall Extract from P. indica (PiCWE)

PiCWE was prepared using the protocol of Anderson-Prouty and Albersheim [2] 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 assays.

2.4. Hydrogen peroxide (H_2O_2) assay (H_2O_2 assay kit; ab 102500; Abcam, USA)

The measurement of H_2O_2 was done using the H_2O_2 assay kit. The kit is a highly sensitive and simple colorimetric and fluorometric assay for measuring H_2O_2 in biological samples. In the presence of Horse Radish Peroxidase (HRP), the OxiRed Probe reacts with H_2O_2 to produce product with colour (λ max = 570 nm). The detection limit can be as low as 2 pmol per assay (40 nM concentration) of H_2O_2 in the sensitive fluorometric assay.

2.4.1. Preparation of samples

Hundred mg of leaf tissues from each treatment were ground into fine powder using liquid nitrogen and dissolved in 2 ml of double distilled water. The thoroughly mixed sample was centrifuged at 5000 g for 5 min and the supernatant was taken. This partially crude preparation can be used for this assay. 2–50 µl of sample was added to each well and adjusted the volume to 50 µl using the assay buffer.

2.4.2. H_2O_2 standard graph for the colorimetric assay

Ten µl of 0.88 M H_2O_2 standard was diluted into 870 µl of distilled water to generate 10 mM H_2O_2 standard, and then 10 µl of 10 mM H_2O_2 standard was diluted with 990 µl of distilled water to generate 0.1 mM H_2O_2 standard. 0, 10, 20, 30, 40 and 50 µl of the 0.1 mM H_2O_2 standard was added to 96-well plate in duplicates to generate 0, 1, 3, 4 and 5 nmol/well of H_2O_2 standard.

Reaction mix: For each well, a total of 50 µl was prepared which includes 46 µl of assay buffer, 2 µl of OxiRed probe solution and 2 µl of HRP solution. 50 µl of the reaction mix was added to each test samples and H_2O_2 standards. After mixing thoroughly the 96-well plate was incubated at RT for 10 min. The OD at 570 nm was measured using a microplate reader.

2.5. Determination of total antioxidant capacity (total antioxidant capacity assay kit; ab65329; Abcam, USA)

2.5.1. Preparation of trolox standard

The lyophilized Trolox standard provided in the kit was dissolved in 20 µl of DMSO by vortexing. Once dissolved, 980 µl of distilled water was added and mixed well to get a 1 mM solution. Following reconstitution, aliquots were stored at −20 °C. For the experiment, the Trolox standard graph was prepared by adding 0, 4, 8, 12, 16 and 20 µl of the Trolox standard to individual wells and adjusted the total volume in each well to 100 µl with double distilled water to give 0, 4, 8, 12, 16, 20 nmol of Trolox standard.

2.5.2. Preparation of samples

Hundred mg of leaf tissues from each treatment (*P. indica* colonized and PiCWE treated ones taken at different time intervals) were ground into fine powder using liquid nitrogen and dissolved in 2 ml of double distilled water. The thoroughly mixed sample was centrifuged at 5000 g for 5 min and the supernatant was taken. This partially crude preparation can be used for this assay.

2.5.3. Preparation of working solutions and procedure

The method of preparations was followed as per the kit instructions. The absorbance was read at 570 nm using the microplate reader (Bio-Rad, Model 680, USA). Standard graph was prepared by plotting the absorbance at 570 nm as a function of Trolox concentration. The sample antioxidant Trolox equivalent concentrations were determined as per the equation mentioned in the kit.

2.6. Quantitation of total phenols

2.6.1. Sample preparation

Portions of 100 mg of leaf tissues were ground into fine powder using liquid nitrogen. 2 ml of 80% methanol was used for the extraction of phenolic compounds by macerating at 15 °C at orbital shaker (200 rpm) for 24 h. The samples were centrifuged for 10 min at 4000 g. The liquid phase was collected after centrifugation and filtration using 0.2 µ filter and the procedure was repeated three times. The pooled supernatant was again extracted with chloroform to remove chlorophyll