Microbiological Research 212–213 (2018) 17–28

Contents lists available at ScienceDirect

Microbiological Research

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

Bacterial endophytes modulates the withanolide biosynthetic pathway and physiological performance in *Withania somnifera* under biotic stress

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ARTICLE INFO

Keywords:
Endophytes
Bacillus amyloliquefaciens
Pseudomonas fluorescens
Biotic stress
A. alternata
Withania somnifera
Withanolides

ABSTRACT

Despite the vast exploration of endophytic microbes for growth enhancement in various crops, knowledge about their impact on the production of therapeutically important secondary metabolites is scarce. In the current investigation, chitinolytic bacterial endophytes were isolated from selected medicinal plants and assessed for their mycolytic as well as plant growth promoting potentials. Among them the two most efficient bacterial endophytes namely *Bacillus amyloliquefaciens* (MPE20) and *Pseudomonas fluorescens* (MPE115) individually as well as in combination were able to modulate withanolide biosynthetic pathway and tolerance against *Alternaria alternata* in *Withania somnifera*. Interestingly, the expression level of withanolide biosynthetic pathway genes (3-hydroxy-3-methylglutaryl co-enzyme A reductase, 1-deoxy-D-xylulose-5-phosphate reductase, farnesyl diphosphate synthase, squalene synthase, cytochrome p450, sterol desaturase, sterol Δ-7 reductase and sterol glycosyl transferases) were upregulated in plants treated with the microbial consortium under *A. alternata* stress. In addition, application of microbes not only augmented withaferin A, withanolide A and withanolide B content (1.52–1.96, 3.32–5.96 and 12.49–21.47 fold, respectively) during *A. alternata* pathogenicity but also strengthened host resistance *via* improvement in the photochemical efficiency, normalizing the oxidized and non-oxidized fraction, accelerating photochemical and non-photochemical quantum yield, and electron transport rate. Moreover, reduction in the passively dissipated energy of PSI and PSII in microbial combination treated plants corroborate well with the above findings. Altogether, the above finding highlights novel insights into the underlying mechanisms in application of endophytes and emphasizes their capability to accelerate biosynthesis of withanolides in *W. somnifera* under biotic stress caused by *A. alternata*.

1. Introduction

It is widely known that the quality and quantity of herbal drugs are influenced through native geographical pattern (Liu et al., 2015), their genetic information (Ganie et al., 2015) along with assessable form of nutrients present in the soil (Quan and Liang, 2017). Therefore, several researchers have paid a lot of attention to explore the native plants along with their genetic variability for advance development (Nag et al., 2015; Panda et al., 2015). However, the study of bacterial endophytes and their impact on the synthesis of pharmaceutically crucial herbal drugs were not much explored. The application of endophytes leads to better understanding of plant-microbe interactions as well as highlights the interplay during the production of herbal compounds of medicinal importance. There are enormous group of microbes interacting with host plants to make a symbiotic association, serving as a valuable micro-ecosystem for plant health management (Andreote et al., 2014). Thus the understanding of host-endophyte interactions may facilitate the enhanced production of crucial herbal drugs by the application of potent endophytes.

The colonization of bacterial endophytes in the host plant is not a chance event as plants secretes several antimicrobial compounds i.e. saponins etc., essential oils which are part of natural selection system and comes from co-evolution and genetic adaptability to overcome itself against several biotic and abiotic threatening issues. In response, bacterial endophytes produce several detoxifying enzymes i.e. protease, cellulase etc. for colonization. As these bacterial endophytes starts colonizing within the plants, a symbiotic association is established with the host. Throughout the co-existence, they share the genetic

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https://doi.org/10.1016/j.micres.2018.04.006
Received 12 October 2017; Received in revised form 22 March 2018; Accepted 21 April 2018
Available online 22 April 2018
0944-5013/

information to ensure the beneficial effects to each other metabolically, genetically and physiologically for advanced development (Schmidt et al., 2014). The host plant facilitates endophytes with nutrients, minerals and favorable condition to ensure its growth and development, whereas, in response microbes benefit the host by enhancing disease resistance, inducing growth along with the accumulation of plant bioactive compounds (Singh and Gaur, 2016). The ability to synthesize plant bioactive compounds by bacterial endophytes may be as a result of their interaction at genetic level. Recently, few reports have demonstrated that endophytes positively affect the flavor of fruiting bodies, secondary metabolites and overall plant growth (Verginer et al., 2010; Andreote et al., 2014; Singh and Gaur, 2016). Furthermore, endophytes performs a decisive role in plant health management *via* solubilization of phosphate, secretion of various antimicrobial substances, different phytohormones (IAA and GA_3), siderophores, as well as inhibit the growth of various phyto pathogens by competing for nutrients and colonization.

Withania somnifera (L.) Dunal (commonly known as Ashwagandha or Indian ginseng) is erect, evergreen, perennial and tomentose shrub and serves as most important medicinal plant over 3000 years. Due to its diverse therapeutically important compounds it is mentioned in ancient books of Unani and Ayurvedic systems as well as in the modern monographs of World Health Organization (WHO) (Sen and Chakraborty, 2017). Leaves and roots of *W. somnifera* are rich sources of the secondary metabolites in the form of alkaloids, withanolides, glycowithanolides, flavanol glycosides, steroidal lactones and polyphenolics which are well known for anti-arthritic, anti-ageing, anticancer, anti-inflammatory, immunoregulatory, chemoprotective, cardioprotective and helps to cope up from neurodegenerative disorders (Pingali et al., 2014; Rai et al., 2016). This plant is highly susceptible to various fungal phytopathogens at wild and cultivated conditions such as *Alternaria alternata, Fusarium oxysporum*, *Myrothecium roridum* etc. among them, *A. alternata* causal organism of leaf spot disease is considered as the most prominent one and causing substantial harm on bioactive content of plant (Pati et al., 2008; Sharma et al., 2014). It has been also reported that the association of *A. alternata* with *W. somnifera* could be the reason of serious lung infection in humans (Chowdhary et al., 2016). Therefore, the use of infected tissues for therapeutic applications is prohibited as per guidelines of World Health Organization (WHO, 2011). Moreover, the progression of leaf spot disease deteriorates the plant tissues at cellular level resulting plant cell death *via* affecting the fundamental processes in plant such as photosynthesis as well as C:N ratio. It drastically reduced the withanolide content by 76.3% (Shivanna et al., 2014). Several chemical based toxic substances were applied to manage *A. alternata* infection but due to numerous negative effects on human, the application of these chemical substances have been restricted (Nicolopoulou-Stamati et al., 2016). While, the microbe mediated eco-friendly and non-hazardous approach to mitigate the adverse impact of leaf spot disease on withanolide biosynthesis along with disease reduction is not yet reported.

With an ever increasing demand of secondary metabolites from *W. somnifera*, efforts are needed to find an eco-friendly potent antagonist to protect it from various fungal phytopathogens which are hampering the secondary metabolites production. Therefore, the present work is focused on the application of potent bacterial endophytes to diminish the pathogenicity of leaf spot disease in *W. somnifera* and their impact on withanolide biosynthetic pathway.

2. Methods and materials

2.1. Isolation of bacterial endophytes from plant samples

Healthy medicinal plant samples (3 plants of each sample) of *Rauvolfia serpentina, Gymnema sylvestre, Stevia crenata, Bacopa monnieri, Andrographis paniculata* and *Withania somnifera* were collected from experimental fields of CSIR- National Botanical Research Institute (Banthara unit), (26° 55′ N, 80° 59′ E), Lucknow, India during June (2013) and stored in sterilized polythene bags at 4 °C. Plants were selected on the basis of its immense therapeutic potentials. Different tissues (leaf, stem and root) of plants were subjected to the endophyte isolation within 24 h after sampling.

2.2. Enrichment and isolation of endophytes

Samples were washed under running tap water and surface sterilized with 70% ethanol and 4% sodium hypochlorite (NaOCl) to eliminate the surface soil and adherent epiphytes completely. The surface sterilized sections of plants were homogenized with 0.85% NaCl and enriched with 1% aqueous solution of colloidal chitin (1gm colloidal chitin in 100 ml saline) aseptically (Ralph Berger and Reynold, 1958). The suspensions were incubated at 28 ± 2 °C, on 150 rpm for 2 weeks. Different dilutions of suspension were spread onto colloidal chitin agar (CCA) and incubated at 28 ± 2 °C for 2 days. After incubation, bacterial colonies growing out with clearing zone were streaked on CCA plates to obtain pure bacterial cultures (Hoster et al., 2005).

2.3. Efficiency of surface sterilization

Efficacy of surface sterilization was evaluated by imprinting the surface sterilized small sections of plant tissue on Nutrient and CCA agar medium (Qin et al., 2009). Simultaneously, autoclaved distilled water used for the final washing of surface sterilization procedure was also spread on both medium (Schulz et al., 1993). Both set up were incubated at 28 ± 2 °C for 2 days and microbial growth was observed to ensure the effectiveness of surface sterilization.

2.4. Characterization of endophytes

Chitinase activity of bacterial endophytes was estimated by the 3,5-dinitrolsalysalic acid (DNS) method as per the earlier described protocol (Gupta et al., 1995). The standard curve of chitinase activity was prepared by diluting different concentration of N-acetyl-D-glucosamine (GlcNAc) (Sigma) in 0.1 M citrate buffer (pH 7.0) to obtain desired concentration. Subsequently, bacterial endophytes were tested qualitatively for ammonia production (Cappuccino and Sherman, 1992), HCN production (Bakker and Schippers, 1987), cellulase activity (Zhou et al., 2004), protease activity (Vermelho et al., 1996), pectinolytic activity (Soares et al., 1999), gelatinase activity (Esteves et al., 2014), and starch hydrolysis (Rohban et al., 2009).

2.5. Plant growth promoting traits of endophytes

For phosphate solubilization activity, freshly grown isolates were spotted on NBRIP agar plates containing tri-calcium phosphate (TCP; insoluble inorganic phosphate source) and bromophenol blue (pH indicator) (Nautiyal, 1999). Culture containing NBRIP agar plates were incubated at 28 ± 2 °C for 48–72 h. Isolates were screened out on the basis of the development of clear halo (Kumar et al., 2012). Subsequently, the ability of solubilization of tri-calcium phosphate in broth culture was quantitatively estimated as described previously (Mehta and Nautiyal, 2001). Endophytes were assayed for siderophores production on the chrome azurol S agar medium (Schwyn and Neilands, 1987). Bacterial isolates were spotted on Chrome azurol S agar plates followed by incubation at 28 ± 2 °C for 48–72 h. Clear orange color zone around the colonies indicated the production of siderophore. Different type of siderophore i.e., Catechol-type and hydroxamate type were assessed by Arnow's method (Arnow, 1937) and Csaky test (Csaky, 1948) respectively to determine the functional groups of siderophores. Quantitative analysis of IAA at different concentrations of L-tryptophan (Basal, 0 mg ml^{-1}; 2 mg ml^{-1}; and 5 mg ml^{-1}) and gibberellic acid production by bacterial isolates was evaluated by the previous methods