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Plant growth promoting rhizobacteria (PGPR) and entomopathogenic fungus bioformulation enhance the expression of defense enzymes and pathogenesis-related proteins in groundnut plants against leafminer insect and collar rot pathogen

G. Senthilraja [a], T. Anand [a,*], J.S. Kennedy [b], T. Raguchander [a], R. Samiyappan [a]

[a] Department of Plant Pathology, Centre for Plant Protection Studies, Tamil Nadu Agricultural University, Coimbatore 641 003, India
[b] Department of Agricultural Entomology, Centre for Plant Protection Studies, Tamil Nadu Agricultural University, Coimbatore 641 003, India

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ABSTRACT

The bioformulation of *Pseudomonas fluorescens* (Pf1 and TDK1) and *Beauveria bassiana* (B2) strains was evaluated individually and in combinations with and without chitin for their efficacy against leafminer insect and collar rot disease and the effect of the interaction between *Pseudomonas*, *Beauveria* and groundnut leafminer insect and collar rot pathogen in the expression of defense enzymes and pathogenesis-related proteins (PR-proteins) in groundnut. Among the various bioformulations, B2 + TDK1 + Pf1 (amended with or without chitin) formulation significantly reduced the incidence of leafminer and collar rot disease when compared to untreated control. A significant increase in the enzymatic activity of phenylalanine ammonia-lyase, peroxidase, polyphenol oxidase, chitinase, β-1,3-glucanase, superoxide dismutase, catalase, lipoxygenase, and phenolics in groundnut plants treated with B2 + TDK1 + Pf1 bioformulation (amended with or without chitin) and challenge inoculated with *Aproaerema modicella* and *Sclerotium rolfsii*. Native gel electrophoresis also revealed the expression of more isoforms of pathogenesis-related proteins and other defense enzymes *viz*., polyphenol oxidase and superoxide dismutase in plants treated with B2 + TDK1 + Pf1 mixture challenged with *A. modicella* and *S. rolfsii*. The present study reveals that sustained and timely induction and accumulation of these defense enzymes and PR-proteins enhance the resistance in groundnut against leafminer insect and collar rot disease.

1. Introduction

Groundnut (*Arachis hypogaea* L.) is a major oilseed crop and is affected by several insect pests and diseases. Among the pests, the leafminer (*Aproaerema modicella* Dev.) is one of the most important pests in the southern parts of India. Yield loss was reported up to 40–60% in India [1] and in some cases it will extend up to 92%. Regarding diseases, collar rot and seedling blight caused by *Sclerotium rolfsii* Sacc. is an important disease and due to that the yield loss was estimated to range from 10 to 80% [2].

The management of groundnut leafminer insect and collar rot disease has been almost exclusively based on the application of chemical pesticides. Several effective pesticides have been recommended for use against these insect pests and pathogen, but they are not considered to be long-term solutions, due to concerns of expense, exposure risks, residue persistence, elimination of natural enemies and other health and environmental hazards. Therefore, the need for alternative methods of control of leafminer insect and collar rot disease has become vital. Unfortunately there is no eco-friendly viable practice currently available for this purpose.

The utilization of a plant's own defense mechanisms is an attractive area of research practiced all over the world to manage plant pests and diseases. Plants treated with plant growth promoting rhizobacteria (PGPR) have latent defense mechanisms against pests and pathogens that can be systemically activated upon exposure of plants to stress or infection by pathogens [3]. This phenomenon is called induced systemic resistance (ISR) [4]. The mechanism operates through the activation of multiple defense compounds at sites distant from the point of pest and pathogen attack [5]. These induced defense responses are regulated by a network of interconnecting signal transduction pathways in which salicylic acid (SA), jasmonic acid (JA) and ethylene (ET) play key roles [6–8].

* Corresponding author.
E-mail address: barathiana@yahoo.com (T. Anand).

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Induced resistance by PGPR is associated with the accumulation of PR proteins *viz*., endochitinases, β-1,3-glucanases and peroxidase [9], which are induced during pathological or related situations. They are not only accumulated locally in the infected leaves but also induced systemically associated with the development of systemic induced resistance against further infection by pathogens [10,11]. Of late, experiments have proven that ISR-mediated resistance by plant growth promoting fluorescent pseudomonads can protect the plants from pest attack by activating defense genes encoding chitinase, proteinase inhibitors and lipoxygenase and expression of stress-related proteins [12,13]. These enzymes and defense molecules have direct and indirect effects against insect pests, such as affecting the suitability of food, which in turn affects the morphogenesis of the insect pest [14,15]. Thus, bacterial strains with ISR activity may increase the possibility of achieving a better management of pests and diseases [15–17].

The biocontrol efficacy of fluorescent pseudomonads can be further increased by mixing two or more strains of *Pseudomonas* spp. [15,16]. Further, the application of biocontrol agents along with chitin has great importance in plant disease and insect management [18,19]. Chitin amendment in the medium has increased the chitinase production which in turn showed increased biocontrol activity against insect pests and plant pathogens [20,21]. In all these studies, the entomopathogenic fungi and PGPR have been tested either in controlled or under field conditions either for diseases or insects control alone. However, no attempts have been made for the management of leafminer insect and collar rot disease by using the mixtures of both entomopathogenic fungus and PGPR strains and to understand the mechanisms of disease resistance induced by entomopathogenic fungus and PGPR strain mixtures. The objectives of the present study are (1) to evaluate the different entomopathogenic fungus and PGPR strain mixtures against leafminer insect and collar rot disease under glasshouse conditions and (2) to elucidate the mechanisms involved in pest and disease resistance by the mixtures of entomopathogenic fungus and PGPR strains in groundnut plants against leafminer insect and collar rot pathogen.

2. Materials and methods

2.1. Plant materials, bioagents and pathogen

Susceptible groundnut cultivar VRI 2 was used in all experiments for evaluating the efficacy of *Beauveria bassiana* and *Pseudomonas fluorescens* consortia against groundnut leafminer insect and collar rot disease. *P. fluorescens* strains TDK1 and Pf1 [16,22,23] and *B. bassiana* strain B2 and B4 [24,25] studied as biocontrol agents against various insect pests and diseases were obtained from the Culture Collection Section, Department of Plant Pathology, Tamil Nadu Agricultural University (TNAU), India. Strains of *B. bassiana* and *P. fluorescens* used in this study were compatible with each other (data not shown). The virulent collar rot pathogen *S. rolfsii* was isolated from groundnut plants showing typical collar rot symptom.

2.2. Preparation of talc-based formulation of biocontrol agents

2.2.1. Entomopathogenic fungal isolates

B. bassiana isolates (B2 and B4) were multiplied in the molasses yeast medium (30 g molasses, 5 g yeast and 1 l water) (for chitin based formulation the medium was amended with 1% colloidal chitin). After multiplication, the broth containing 13×10^7 cfu ml^{-1} in the flask was mixed with talc at 1:2 ratio (500 ml:1 kg). To the mixture, 5 g of carboxymethyl cellulose (CMC) was added as sticker and dried in shade for 72 h, powdered and stored in polypropylene bags [26]. The population of *B. bassiana* during application was 1×10^8 cfu g^{-1} of talc powder.

2.2.2. PGPR strains

The talc-based formulation of each of the individual bacterial strains was prepared with some modification of method developed by Vidhyasekaran and Muthamilan [27]. A loopful of *P. fluorescens* strain was inoculated into the KB broth and incubated in a rotary shaker at 150 rpm for 48 h at room temperature (28 ± 2 °C). One kilogram of talc powder was taken in a sterilized metal tray and its pH was adjusted to neutral by adding calcium carbonate at the rate of 15 g kg^{-1}. Ten gram of CMC was added to 1 kg of talc and mixed well and the mixture was autoclaved for 30 min on each of two consecutive days. The 400 ml of 48 h grown bacterial suspension containing 9×10^8 cfu ml^{-1} were mixed with carrier-cellulose mixture under aseptic conditions. After drying (approximately to 35% moisture content) for overnight, it was packed in polypropylene bag, sealed and stored at room temperature (28 ± 2 °C). At the time of application, the population of *P. fluorescens* in the formulation was 3×10^8 cfu g^{-1} of talc powder.

2.2.3. Mixtures of entomopathogenic fungus and PGPR strains

Bioformulations containing mixtures of fluorescent pseudomonads were prepared by growing the *Pseudomonas* strains separately in KB broth, mixing equal volumes of each and finally blending with talc powder, calcium carbonate and CMC. Same method was followed to the *Pseudomonas* strains with *B. bassiana* strain mixtures. The talc-based bioformulation mixtures were stored at 28 ± 2 °C and used for further applications.

2.2.4. Chitin amendments with talc-based formulations

Five gram of crab shell chitin (Sigma, USA) was slowly added into 100 ml of cold 0.25 N HCl with vigorous stirring and kept overnight at 4 °C. The mixture was filtered through glass wool into 200 ml of ice cold ethanol at 4 °C with rapid stirring. The resultant chitin suspension was centrifuged at 10,000 rpm for 20 min and the chitin pellets were washed repeatedly with distilled water until the pH became neutral [28]. The concentration was adjusted to 10 mg ml^{-1} and added to KB broth (1%, v/v). Then the liquid medium (containing chitin) was autoclaved and the respective *B. bassiana* and *P. fluorescens* strains were allowed to grow in the chitin amended medium for 48 h. Finally, the chitin amended talc-based formulations were prepared as described earlier.

2.3. Mass rearing of leafminer

Initial culture of *A. modicella* was established in the laboratory by collecting larvae from groundnut plants. The isogenic culture was initiated from the larvae collected from the field in Department of Oilseeds, Centre for Plant Breeding and Genetics, TNAU, Coimbatore. These larvae were reared separately in the clean specimen glass Petri dish (15 × 1.5 cm) and fresh leaves were provided daily in each Petri dish as a food for the larvae. After pupation, pupae formed in the leaves were collected and transferred into Petri dishes individually for the emergence of adults.

2.4. Glasshouse studies

2.4.1. Effect of entomopathogenic fungus and fluorescent pseudomonad strains against leafminer insect and collar rot disease

Pot culture studies were conducted to test the efficacy of *B. bassiana* and *P. fluorescens* strain mixtures (with and without chitin amendment) as seed, soil and foliar applications separately in controlling collar rot disease and leafminer incidence in groundnut. The virulent isolate of *S. rolfsii* was mass multiplied in the sand-maize medium, mixed with the sterilized pot soil @ 15 g kg^{-1} of soil and filled in earthen pots. Five gram of the talc-based bioformulation mixture was given as soil application per