Biological Control 63 (2012) 87–97

Contents lists available at SciVerse ScienceDirect

Biological Control

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

Improvement of peanut rhizobial inoculant by incorporation of plant growth promoting rhizobacteria (PGPR) as biocontrol against the seed borne fungus, *Aspergillus niger*

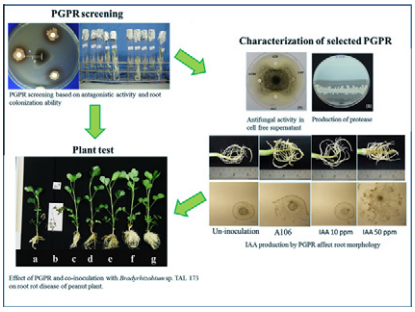
Watcharin Yuttavanichakul [a], Pruksa Lawongsa [b], Sopone Wongkaew [c], Neung Teaumroong [a], Nantakorn Boonkerd [a,*], Nobuhiko Nomura [d], Panlada Tittabutr [a,*]

[a] School of Biotechnology, Institute of Agricultural Technology, Suranaree University of Technology, Nakhon Ratchasima 30000, Thailand
[b] Department of Plant Science and Agricultural Resources, Faculty of Agriculture, Khon Kaen University, Khon Kaen 40002, Thailand
[c] School of Crop Production Technology, Institute of Agricultural Technology, Suranaree University of Technology, Nakhon Ratchasima 30000, Thailand
[d] Graduate School of Life and Environmental Sciences, University of Tsukuba, Tsukuba, Ibaraki 305-8572, Japan

HIGHLIGHTS

- We have selected PGPR isolates that could inhibit *Aspergillus niger*.
- Some strains have more than one antifungal mechanism, and might produce a substance in the lipopeptides group.
- Inoculation of PGPR affected root morphology; it was similar to plants treated with some concentration of IAA.
- The antagonistic activities of PGPR are similar to the effect of using 90 ppm carbendazim on root rot disease.
- PGPR could promote plant growth, and co-inoculation with *Bradyrhizobium* could protect plant from *A. niger* infection.

GRAPHICAL ABSTRACT



ARTICLE INFO

Article history:
Received 7 May 2011
Accepted 17 June 2012
Available online 30 June 2012

Keywords:
Aspergillus niger
Biocontrol
Bradyrhizobium
Peanut
PGPR
Rhizobial inoculant

ABSTRACT

The inhibition of *Aspergillus niger* that causes root rot diseases in peanut (*Arachis hypogaea* L.) was investigated by using 765 bradyrhizobial and 350 soil-isolated plant growth promoting rhizobacteria (PGPR) strains as biological controllers. Only 11 PGPR isolates were found to be able to inhibit *A. niger* growth. Based on their ability to inhibit *A. niger* growth and root colonization, the best four PGPR isolates, A20, A45, A62, and A106, were selected, and their 16S rRNA sequences were highly homologous to *Bacillus megaterium*, *B. subtilis*, *B. subtilis* subsp. *subtilis*, and *Pseudomonas* sp., respectively. The production of a lytic protease enzyme was detected in A20, A45, and A62, but not in A106. Some antifungal activities were clearly found in cell-free supernatants of A20 and A62. Interestingly, the antifungal activity of isolates A45 and A62 was proteinase K resistant. All PGPR isolates could produce an auxin (indole-3-acetic acid, IAA) hormone and biofilms. IAA produced from PGPR isolates could clearly promote peanut root growth. When either isolate A20 or A45 (10^8 cells per ml) was co-inoculated with *Bradyrhizobium* sp. TAL 173 (10^8 cells per ml), the peanut root rot disease caused by *A. niger* (10^5 and 10^6 spores per seed) could be inhibited. Incorporating rhizobia with selected PGPR increases nitrogen fixation and reduces fungicide usage in peanut, providing an appropriate approach for sustainable agriculture.

* Corresponding authors. Fax: +66 44 224154.
E-mail addresses: nantakon@sut.ac.th (N. Boonkerd), panlada@sut.ac.th (P. Tittabutr).

1049-9644/$ - see front matter © 2012 Elsevier Inc. All rights reserved.
http://dx.doi.org/10.1016/j.biocontrol.2012.06.008

1. Introduction

Peanut or groundnut (*Arachis hypogaea* L.) is an important legume crop. Asia is responsible for 55.2% of global peanut production. China is the largest producer of peanut with 37.5% of overall world production (Dutta et al., 2011). Normally, the peanut seed is coated with rhizobial inoculants before sowing, which fix nitrogen from the air to plant and soil. However, the yield is low due to diseases caused by various microorganisms. The crown rot diseases of peanut caused by *Aspergillus niger* and *A. flavus* are the most important ones in both temperate and tropical countries. The diseases are caused by seed borne pathogens that can survive in infected peanut seeds (Magnoli et al., 2006). *A. niger* and *A. flavus* can produce harmful mycotoxins, ochratoxin, and aflatoxin. Those mycotoxins are human carcinogens and they can accumulate in the meat of animals (Hussein and Brasel, 2001). Thus, seed treatment with chemical fungicides, such as carbendazim (methyl-2-benzimidazol carbamate), is used to protect the seed from pathogenic organisms before sowing. Carbendazim is a systemic benzimidazole fungicide that plays a very important role in plant disease control. It is a derivative of other fungicides such as benomyl, and is applied world-wide on numerous crops (tobacco, fruit, vegetables, cereals, etc.) to control fungi that cause plant diseases (Medina et al., 2007). However, non-target soil microorganisms, such as beneficial bacteria as well as coated rhizobial inoculants, are also affected by these chemical substances (Castro et al., 1997). Although the World Health Organization (WHO) has classified carbendazim as unlikely to present a hazard in normal use, carbendazim may cause reproductive toxicity, or may be a carcinogen, mutagen, or have negative effects on humans. Moreover, there is a considerable interest in finding alternatives to chemical pesticides for suppression of soil borne plant pathogens due to human health and environmental concerns (Haggag, 2007). Therefore, the use of microorganisms to control plant diseases offers an attractive alternative to the use of synthetic chemicals (Roberts et al., 2005).

Rhizosphere bacteria that exhibit root colonization and exert beneficial effects on plants are termed plant growth promoting rhizobacteria (PGPR) (Karthikeyan et al., 2010). PGPR, in combination with efficient rhizobia, could improve growth and nitrogen fixation in the nodules of legumes (Tilak et al., 2006). It has been reported that *Rhizobium trifolii* has potential as a biological control agent against root rot of *Trifolium subterraneum* seedlings caused by *Phytophthora clandestine* (Simpfendorfer et al., 1988). *In vitro* tests of *R. meliloti* inhibited growth of *Macrophomina phaseolina, Rhizoctonia solani*, and *Fusarium solani*, while *Bradyrhizobium japonicum* inhibited growth of *M. phaseolina* and *R. solani* (Ehteshamul-Haque and Ghaffar, 1993). PGPR could also elicit plant defenses (Van Loon and Glock, 2004) and antagonize or prevent growth of phytopathogens or deleterious microorganisms (Kloepper et al., 2004). The biocontrol agents *Pseudomonas fluorescens*, *Trichoderma virens*, and *Bacillus subtilis* showed complete inhibition of *A. flavus* growth (Reddy et al., 2009), and *Paenibacillus polymyxa*, or *Pseudomonas aeruginosa* strains GPS 21, GSE 18, GSE 19, and GSE 30 could control crown root rot disease in peanut (Haggag and Timmusk, 2008; Kishore et al., 2007).

PGPR have the potential to promote plant growth, enhance legume plant nodulation with *Rhizobium* spp., and inhibit the growth of plant pathogens, and rhizobial inoculants have been used for controlling diseases in peanut caused by *A. flavus* or *A. niger* (Ahmad et al., 2008; Moretti et al., 2008). However, PGPR have not been co-inoculated with *Bradyrhizobium* spp. for enhancement of peanut growth and inhibition of the growth of plant pathogenic fungi *A. niger* and *A. flavus* on peanut. Therefore, the aims of this study were to obtain peanut rhizobia and PGPR antagonistic against *A. niger* and to investigate the symbiotic efficiency and biocontrol activity of selected isolates on peanut infected with *A. niger*. A rhizobial inoculant containing a mixture of *Bradyrhizobium* and PGPR could be used instead of fungicide to coat peanut seeds before sowing.

2. Materials and methods

2.1. Bacterial strains

A total of 265 peanut bradyrhizobial strains and commercial *Bradyrhizobium* sp. TAL 173 were obtained from the Department of Agriculture (DOA), Thailand, while 500 rhizobial strains were isolated directly from 500 nodules derived from several peanut plants grown in the field according to the method of Somasegaran and Hoben (1994). *P. fluorescens* CHAO, *P. fluorescens* F113, and an additional 350 PGPR strains were obtained from the School of Biotechnology, Suranaree University of Technology, Thailand. These PGPR were isolated from soil from organic farms in different provinces of Thailand, including Chiangmai (18°47′25″N/98°58′54″E), Lampang (18°17′25″N/99°30′25″E), Nakhon Sawan (15°41′0″N/100°7′0″E), Saraburi (14°51′0″N/100°91′0″E), and Nakhon Ratchasima (14°58′0″N/102°7′0″E). Rhizobia and PGPR isolates were maintained on Congo Red Yeast Extract Mannitol Agar (CRYMA) (Somasegaran and Hoben, 1994) and LG (N-free) media (Lipman, 1904), respectively.

2.2. Fungal strains and in vitro antagonistic activity

The fungi *A. niger* and *A. flavus* were obtained from stock cultures at the Microbial Culture Collection and Application Research Unit, Institute of Science, Suranaree University of Technology, and their disease symptoms were confirmed on peanut (*Arachis hypogaea*, Tainan 9) growing in Leonard jars placed on a shelf with light intensities of 639 $\mu E/m^2/s$, 16-h-day and 8-h-night cycle at 25 ± 2 °C (light room condition). Both fungi were pure cultured from spores from root rot and crown rot disease on peanut used in this study. The *in vitro* inhibition of mycelial growth of *A. niger* and *A. flavus* by the bacterial isolates was tested using the dual culture technique as described by Landa et al. (1997). Triplicate 20 µl drops of 10^8 cells per ml suspension of each PGPR were placed equidistantly on sterile paper at the margins of Nutrient Agar (NA) plates (fungus and bacteria both grow well in this medium). Then, a 4 mm agar plug from a fresh PDA culture of *A. niger* was placed at the center of the plate. Plates were incubated at 28 °C for 7 days and the radii of the fungal colony towards and away from the bacterial colony were measured. The percentage inhibition of mycelial growth (% Img) was calculated using the following formula: (see Graphical abstract).

$$\%\mathrm{Img} = (A - B) \times 100/A$$

where *A* is the distance from the center of fungal growth to the edge of bacterial growth and *B* is the radius of fungal mycelia growth.

2.3. In vitro root colonization

The isolates which showed antagonistic activity in the dual culture assays were tested for their ability to colonize peanut roots *in vitro*, using a modification of the methods of Patten and Glick (2002). Surface sterilized and pre-germinated peanut seeds were grown in sterile test tubes (one seed per tube) containing Hoagland's solution (Idris et al., 2007), and a 1 ml aliquot of each PGPR isolate (10^8 colony forming unit (cfu) per ml) was inoculated into each tube. The tubes were incubated at room temperature for 1 h