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Regulation of inducible enzymes and suppression of anthracnose using submicron chitosan dispersions

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ABSTRACT

The mechanism of suppression of *Colletotrichum gloeosporioides* was studied by using submicron chitosan dispersions. *In vitro* and *in vivo* experiments showed that mycelial growth and disease incidence/severity were significantly ($P < 0.05$) suppressed in 600 nm submicron chitosan dispersion at 1% chitosan concentration. Field trials showed that host resistance was stimulated in dragon fruit plants at all the submicron chitosan dispersions (SCD) used. Results showed that the application of SCD significantly enhanced the production of plant-defense related enzymes such as PO, PPO and PAL. A further increase in inducible compounds and PR proteins was observed in plants inoculated with *C. gloeosporioides*. Increase in inducible compounds and delayed symptoms of anthracnose indicated the possible mechanism of induced resistance. However, the resistance decreased with the passage of time which could be due to highly stressed environment of the plants. Therefore, it is suggested that the plants should be sprayed with SCD to sustain the absolute effect.

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1. Introduction

Plants possess a complex set of pre-formed structures and inducible reactions which can successfully protect them against pathogens (Trotel-Aziz et al., 2006). To detect the possible pathogen attack these inducible reactions require the perception of signal molecules, after which the plant generates a wide range of defence mechanisms which can be useful tools of protection against the assaulting pathogens. This plant defence mechanism could involve the stimulation of the phenylpropanoid and fatty acid pathways, or production of defence-specific chemical messengers such as salicylic acid or jasmonates. The accumulation of components with antimicrobial activities such as phytoalexins has also been reported in some plants (Kombrink and Somssich, 1995).

These so-called elicitors have diverse chemical nature and include proteins, peptides, glycoproteins and oligosaccharides (Côté et al., 1998; Klarzynski et al., 2000). Over the past decade, the use of exogenous elicitors to stimulate natural defence responses has become an important tool (Métraux et al., 1991). Some well-defined oligosaccharides, including microbial or algal β-1,3 glucans, chitin and chitosan derived oligomers, have been shown to enhance the non-host plant resistance against pathogens (Côté et al., 1998; Manjunatha et al., 2008).

Dragon fruit (*Hylocereus polyrhizus*) (Weber) Britton & Rose) seeks global attention due to economic value as a source of food and high antioxidative activity resulting from high betacyanin content (Le Bellec et al., 2006). However, in recent years, its growth and yield has been threatened due to anthracnose caused by the fungus *Colletotrichum gloeosporioides* (Masyahit et al., 2008). Currently, synthetic fungicides are widely used to control the disease (Hoa, 2008). However, these chemical pesticides results in several ecological problems. Therefore, eco-friendly and sustainable approach could be a potential method to reduce the damage caused by fungicides and may be economically advantageous when used in disease management programme.

Chitosan could be a possible contender to induce various defence responses in several plants, including phytoalexin and PR proteins (Hadwiger and Beckman, 1980; Agrawal et al., 2002). Consequently, there was inhibition of fungal growth and promotion of

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protection from further infection (Hadwiger and Beckman, 1980). However, negative effects of chitosan on plantlet growth, shoot length and also the death of plantlet were reported when used at higher concentrations (Ait Barka et al., 2004). Therefore, alternatively SCD can be utilised to reduce the phytotoxic effects of chitosan. Moreover, in a recent study by our group it was found that SCD enhanced the antifungal properties of chitosan by activating the efficacy of PR proteins and helped in enhancing the shelf life of tropical fruits (Zahid et al., 2012; Ali et al., 2014). These PR proteins and peroxidases are the key enzymes in cell wall building process and it has been suggested that these peroxidases and inducible enzymes would enhance resistance in various plant species against phytopathogens by constructing a cell wall barrier that may hinder pathogen attack and spread in the plant cells (Taheri and Tarighi, 2011).

Up to our knowledge, no study has been reported on the activation of peroxidases and inducible enzymes via SCD. Therefore, the present study was designed to examine the level of these enzymes and peroxidases by using SCD through 'pathosystem' dragon fruit and *C. gloeosporioides*.

2. Materials and methods

2.1. Materials

Red dragon fruit cuttings were purchased from the local orchard located at Puchong, Selangor, Malaysia. Low molecular weight chitosan (Mw: 50 kDa; 75–85% deacetylated) from crab shell and Span 20 were obtained from Sigma–Aldrich, (USA), while Brij 56, potato Dextrose Agar (PDA) and potato dextrose broth (PDB) were obtained from Merck KGaA, (Darmstadt, Germany).

2.2. Fungus isolation and inoculum preparation

To isolate the fungus small portions of naturally infected dragon fruit stem were surface sterilized with 0.5% sodium hypochlorite solution followed by washing with purified water and air dried. *C. gloeosporioides* isolates were cultured on petri plates having PDA and incubated at room temperature. Once the mycelial growth was observed, the colonies were again cultured on fresh PDA plates to obtain pure cultures. Identification of these isolates was carried out on morphological basis (Banrnett and Hunter, 1972). Koch's postulate was proved true when the disease appeared on the healthy dragon fruit stem after inoculation with *C. gloeosporioides*. Re-isolations on PDA plates were carried out to maintain the inoculum.

2.3. Preparation of conventional chitosan solution and submicron chitosan dispersions

Based on previous results obtained from *in vitro* study by our group only 1.0% chitosan was selected to perform further experiments (Zahid et al., 2012).

Conventional chitosan (CC) solution was prepared by dissolving 1 g of chitosan powder in 100 ml of 0.5% glacial acetic acid. SCD were prepared by an alcohol free nano emulsifying system. Brij 56 and Span 20 were used as emulsifiers. These emulsifiers were mixed in a ratio of 1:1 and used to prepare SCD. A 100 ml of chitosan solution was mixed with 0.1 ml of emulsifier mixture and allowed to sonicate in an ultra sound bath (Model: Bandelin Sonorex, Germany) for different time intervals to obtain different droplet sizes. Size of the droplet was measured by using zetasizer (Zetasizer NanoZS, Malvern, UK) at a scattering angle of 173° (Zahid et al., 2012).

2.4. In vitro antifungal activity of different types CC and SCD against C. gloeosporioides

2.4.1. Inhibition in radial mycelial growth

In vitro antifungal activity of CC and SCD was determined using poisoned food technique (Maqbool et al., 2010). Briefly, petri dishes containing 15 ml of PDA were poisoned with 1.0% CC solution and SCD with different droplet sizes (nm), *i.e.* 200, 600 and 1000 at 1.0% chitosan concentration. After solidification each of plates were seeded with 5 mm diameter of *C. gloeosporioides*. Petri dishes containing PDA only were used as controls. The antifungal assay of emulsifiers (Brij 56 and Span 20) was also carried out alone and in combination to verify their contribution in inhibition in radial mycelial growth. Petri dishes were incubated at 28 ± 2 °C and measurement of radial mycelial growth was taken daily until the mycelia in control dishes reached at the edge.

2.5. Field layout

Diseased free dragon fruit stem cuttings of 1.5 feet were obtained from 2 years old mature fruit bearing plants. Cuttings were grown for two months in a nursery space provided by the University of Nottingham, Malaysia Campus, Malaysia. After 2 months, plants were transplanted in a field plot having dimensions 69 m × 36 m at Farm no B1, Taman Pertanian, Universiti Putra Malaysia, Serdang, Malaysia located at latitude 2.9992°N and longitude 101.7078°E. Pole system was already installed in the field. Planting holes were prepared by digging the soil to a depth of 14 cm and four plants per pole were planted. Irrigation was carried out daily in the evening through sprinkler irrigation system for first two months and later on at three days interval to ensure adequate moisture in the soil. Fertilizer application was scheduled according to the recommendations made by Ali et al. (2014).

Two trials were carried out to ensure the reproducibility of results of this study. First trail was conducted from December, 2011 to September, 2012 while the second trail was conducted from February, 2012 to November, 2012. Plants were allowed to grow in the field until they achieve a length of 1.8 m. Plants were then sprayed with CC and SCD until runoff 24 h prior to inoculation with *C. gloeosporioides*. Plants were inoculated artificially by using sterile sharp toothpick. An artificial wound of approximately 2 mm deep was made by using toothpick and then 20 μl of spore suspension (1×10^6 spores ml^{-1}) was injected into the wounded site by using a sterile syringe needle. Inoculations were made on five stems on each plant and five different spots were inoculated on each stem. Uninoculated plants were also treated in the same manner and served as control (Siddiqui et al., 2009). Extra plants were planted on all the four sides of plants in order to minimise the border effect. Plants were sampled destructively on alternate days until one month.

2.5.1. Assessment of disease incidence (DI%)

DI was assessed on a basis of water soaked necrotic lesions at each sampling time. DI was expressed in percentage (%) and calculated by using the following formula:

$$\text{Disease incidence}\,(\%) = \frac{\text{Number of infected stems}}{\text{Total number of stems assessed}} \times 100$$

2.5.2. Disease severity (DS) and area under disease progress curve (AUDPC)

DS was recorded according to the rating scale of 1–5 after 30 days (where 0 = no disease, 1 = 1–20% disease symptoms, 2 = 21–40% disease symptoms, 3 = 41–60% disease symptoms, 4 = 61–80% disease symptoms and 5 = 81–100% disease symptoms),

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