Scientia Horticulturae 243 (2019) 314–319

Contents lists available at ScienceDirect

Scientia Horticulturae

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

Inhibition in production of cellulolytic and pectinolytic enzymes of *Colletotrichum gloeosporioides* isolated from dragon fruit plants in response to submicron chitosan dispersions

Noosheen Zahid[a,b], Mehdi Maqbool[a,b], Asgar Ali[b,*], Yasmeen Siddiqui[c], Qaiser Abbas Bhatti[d]

[a] Department of Horticulture, Faculty of Agriculture, The University of Poonch, Rawalakot, Azad Jammu and Kashmir, Pakistan
[b] Centre of Excellence for Postharvest Biotechnology, School of Biosciences, The University of Nottingham, Malaysia Campus, 43500 Selangor, Malaysia
[c] Laboratory of Climate-Smart Food Crops, Institute of Tropical Agriculture and Food Security, Universiti Putra Malaysia, Serdang 43400, Selangor, Malaysia
[d] Department of Chemistry, Faculty of Basic and Applied Sciences, The University of Poonch, Rawalakot, Azad Jammu and Kashmir, Pakistan

ARTICLE INFO

Keywords:
AUDPC
β-Galactosidase
β-1, 4-Glucanase
Pectin lyase
Polygalacturonase

ABSTRACT

In vitro and *in vivo* antifungal potential of submicron chitosan dispersions (SCD) was studied in order to investigate the effect of SCD on cell wall degrading enzymes of *Colletotrichum gloeosporioides* and to control anthracnose of dragon fruit under field conditions. Four concentrations of chitosan (0.5, 1.0, 1.5 and 2.0%) were used to prepare submicron dispersions with droplet sizes of 200, 400, 600, 800 and 1000 nm. The highest inhibition in cellulolytic and pectinolytic enzymes was observed with 600 nm droplet size of 1.0% chitosan concentration. In case of *in vivo* studies,the area under disease progress curve (AUDPC) showed a reduction of approximately 66% of disease incidence (DI). The lower level of production of cell wall degrading enzymes in response to SCD resulted in less disease on dragon fruit plants and sustained in increasing the effective resistance against the irresistible disease of anthracnose.

1. Introduction

Colletotrichum is one of the mostdevastating pathogens of crops, causing a serious disease of anthracnose on numerous fruits, vegetables, legumes and cereals (Weir et al., 2012). Dragon fruit (*Hylocereus polyrhizus* (Weber) Britton & Rose; family *Cactaceae*) is an important fruit crop of Malaysia which is affected by anthracnose with yield losses up to 20–80% (Masyahit et al., 2009).

Fungus *Colletotrichum gloeosporioides*, enters plants through natural openings, by mechanical force or wounds on the surface of plants (Kimaru et al., 2018). It is hemibiotrophic and takes nutrients from dead and living cells. It produces several types of cell wall degrading enzymes which play a major role in the infection process and development of symptoms (Kimaru et al., 2018). These enzymes help the fungal hyphae penetrate the mechanical barrier of the plant cell wall (Anand et al., 2008) resulting in death and maceration of infected tissues (Fernando et al., 2001). Cultural practices, such as crop rotation, enhancement of soil quality, water management, growing of resistant varieties, field sanitation, habitat management and chemical fungicides, are commonly used to control the disease (Weir et al., 2012). Cultural or chemical control strategies have not been very effective against this disease (Lin et al., 2017).

The use of natural compounds, such as chitosan, for the control of anthracnose is a novel approach. Chitosan is well known for its versatile antifungal, biodegradable, nontoxic and biocompatible properties (Romanazzi et al., 2002). However, some drawbacks, such as its high viscosity, have been reported (Zahid et al., 2012). Chitosan in the form of submicron dispersions (SCD), which are low in viscosity and penetrate tissues more rapidly, is a novel approach to control anthracnose of dragon fruit plants (Zahid et al., 2014).

To our knowledge no studies have been conducted on the effect of SCD against cell wall degrading enzymes produced by the *C. gloeosporioides*. Therefore, a study was designed to evaluate the efficacy of SCD against the cell wall degrading enzymes produced by *C. gloeosporioides* and to determine the *in vitro* and *in vivo* antifungal activity of SCD against anthracnose.

2. Materials and methods

2.1. Preparation of conventional chitosan (CC) and SCD

Low molecular weight chitosan from crab shell (50 kDa; 75–85%

* Corresponding author.
E-mail address: asgar.ali@nottingham.edu.my (A. Ali).

https://doi.org/10.1016/j.scienta.2018.08.011
Received 8 June 2018; Received in revised form 6 August 2018; Accepted 7 August 2018

deacetylated) was purchased from Sigma-Aldrich, USA. Brij 56 (Merck KGaA, Darmstadt, Germany) and Span 20 (Sigma-Aldrich, USA) were used as emulsifiers.Four concentrations of chitosan (0.5, 1.0, 1.5 and 2.0%) were used to prepare CC and SCD with droplet sizes of 200, 400, 600, 800 and 1000 nm, as described by (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°.

2.2. In vitro extraction of cellulolytic and petinolytic enzymes

2.2.1. Extraction of cellulolytic enzymes

Fifty ml of Czapek's broth (Sigma Aldrich) containing 0.5% carboxy methyl cellulose (w/v) (acting as a sole carbon source) with submicron dispersions of chitosan at 0.5, 1.0, 1.5 and 2.0% was added to 250 ml Erlenmeyer flasks. The flasks containing above mentioned mixtures were inoculated with 9 mm mycelia disc from ten days old culture of *C. gloeosporioides* and incubated for 10 days at room temperature. The contents of the flasks were filtered after 10 days using Whatman's filter (Whatman™ 1001-047 Grade 1, Pore size 11 µm). The 150 ml of filtrate was mixed with 350 ml of 0.1 M potassium phosphate buffer pH 6.8 followed by centrifugation at 5000 x *g* for 20 min at 4 °C. The supernatant was lyophilized and served as the source for enzyme assays.

2.2.1.1. Activity of β-galactosidase. β-galactosidase activity was determined by hydrolysis of the chromogenic substance, *O*-nitrophenyl-β-$_D$-galactoside (ONGP) as a substrate. An extract of 100 µl was mixed with 0.9 ml of Z-buffer and 2 ml of breaking buffer. Where, Z-buffer contained 60 mM $Na_2HPO_4.7H_2O$, 40 mM $NaH_2PO_4.H_2O$, 10 mM KCl and 1 mM $MgSO_4.7H_2O$ adjusted to pH 7. After preparation, the buffer was stored at 4 °C and breaking buffer contained 50 ml of 100 mMTris – HCl (pH 8), 1 ml of 1 mM dithiothreitol and 50 ml of 20% glycerol. Before use, 2.7 ml of 2-mercaptoethanol was added in one litre of the Z-buffer. The mixture was incubated in a water bath at 28 °C for 5 min. ONGP (0.2 ml) was added and the mixture incubated again at 28 °C in a water bath. The reaction was stopped after 30 min by adding 0.5 ml of Na_2CO_3. The hydrolysis was monitored spectrophotometrically at 420 nm using a UV–vis spectrophotometer (Varioskan Flash Multimode Reader, Thermo Fisher Scientific, USA). One β-galactosidase activity unit (U) was defined as the quantity of enzyme required for hydrolysis of 1 µmol of substrate (ONGP) per minute (U mg^{-1} protein) under given experimental conditions. The protein content of the filtrate was measured with bovine serum albumin (BSA) as standard. Specific activity was measured using the formula given by Gueguen et al. (1997).

$$\beta\text{–}galactosidase\ activity = \frac{OD_{420} \times 1.7}{0.0045 \times protein \times extract\ volume \times time}$$

Where, OD_{420} is optical density of product at 420 nm; factor 1.7 corrects the reaction volume; factor 0.0045 was optical density of *O*-nitrophenol at 420 nm.

2.2.1.2. Activity of β-1, 4-glucanase. β-1,4-glucanase activity was measured spectrophometrically by determining the amount of reducing sugars released from 1 g of filter paper (Whatman™ 1001-047 Grade 1) as substrate, using dinitrosalicylic acid as a reagent (Gopinath et al., 2006). Crude extract (0.5 ml) was added to 1 ml of Na-citrate buffer pH 4.8 and incubated at 50 °C for 60 min. After incubation, 3.0 ml of 1.0% 3,5-dinitrosalicylic reagent was added to the reaction mixture and boiled at 100 °C for 5 min, followed by immediate incubation on ice. The amount of reducing sugars produced was determined by a UV–vis spectrophotometer (Varioskan Flash Multimode Reader, Thermo Fisher Scientific, USA) at 540 nm. The enzyme activity was expressed as mg glucose released min^{-1}. Boiled enzyme extract served as a control (Jayaraj and Radakrishnan, 2003). A standard curve was prepared using a glucose stock solution. Specific activity was measured using the following formula after correcting the values with the glucose standard curve.

$$\beta\text{–}1,\ 4\text{–}glucanase\ activity = \frac{0.185}{enzyme\ concentration\ to\ release\ 2.0\ mg\ glucose}$$

Where, factor 0.185 is the optical density of 1.0% 3, 5-dinitrosalicylic acid at 540 nm

2.2.2. Extraction of pectinolytic enzymes

Pectinolytic enzymes were extracted in fifty ml of Czapek's broth (Sigma Aldrich) containing 1.0% citrus pectin (w/v) (acting as a sole carbon source) with submicron dispersions of chitosan at 0.5, 1.0, 1.5 and 2.0% was added to 250 ml Erlenmeyer flasks (Jayaraj and Radhakrishnan, 2003). The supernatant obtained was precipitated by adding 75% chilled acetone and centrifuged for 10 min at 10,000 rpm and 4 °C. The pellets obtained were washed twice with 95% chilled ethanol and used for further determination of enzyme activity (Zahid, 2014).

2.2.2.1. Activity of Polygalacturonase. Pellets obtained after extraction were re-dissolved in 1 ml of 50 mM potassium phosphate buffer at pH 5.5, incubated in a water bath for five hours at 30 °C and absorbance was measured at 556 nm (ε_M = 54176 mol^{-1} l^{-1} cm) with a UV–vis spectrophotometer (Varioskan Flash Multimode Reader, Thermo Fisher Scientific, USA) (Zahid, 2014). One unit of polygalacturonase was defined as the amount of enzyme that liberates 1 µmol of reducing groups per ml at 30 °C using *D*-galacturonic acid as a standard.

2.2.2.2. Activity of pectin lyase. Pellets obtained after extraction were dissolved in a mixture of 1 ml of 50 mM Tris – HCl and 1 ml of 1 mM $CaCl_2$ pH 8.5. One hundred µl of the dissolved sample was mixed with reaction mixture, which contained 200 µl of 1.0% polygalacturonic acid dissolved in 1.0% pectin from citrus and 50 µl of 50 mM Tris – HCl, and incubated at 30 °C for five hours. The absorbance was measured at 550 nm with a UV–vis spectrophotometer (Varioskan Flash Multimode Reader, Thermo Fisher Scientific, USA). One unit of pectin lyase was defined as the amount of enzyme producing one µmol of unsaturated product per ml at 30 °C A standard curve of BovineSerum Albumin was used to determine protein content. Specific activity was defined as U mg^{-1} protein.

2.3. Plant material and experimental site preparation

Size of the experimental area was 87 × 45 m. A system of poles was already installed in the field with poles 2 m high and 3 m apart.Cuttings of 0.5 m in length, from mature, healthy red dragon fruit plants (2 years old), were obtained from a commercial orchard located at Puchong, Selangor, Malaysia. These cuttings were rooted and grown in a shade house at The University of Nottingham, Malaysia Campus. After 2 months, they were planted in the field plot at Farm No.B2, Taman Pertanian University, UPM, Serdang, Malaysia.Soil of the field site was Serdang series (Table 1).Planting holes were prepared by digging the soil to a depth of 14 cm and four plants were planted at each pole.

Rate and schedule of fertilizer application was done according to the recommendations made by Zahid (2014). Organic fertilizer Amino-Q® (1.38% N: 3.02% P_2O_5: 0.98% K_2O: 0.87% MgO: 5.74% CaO) was