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Induced Chitinase and Chitosanase Activities in Turmeric Plants by Application of β-D-Glucan Nanoparticles

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Abstract

The chitinase and chitosanase activities after β-D-glucan nanoparticle (GNP) application turmeric plants (leaves and rhizomes) were measured. Foliar spray of GNP (0.1%, w/v) elicited marked an increase in the activity levels of chitinases and chitosanases. Such a growth of enzyme activities was enhanced by subsequent spraying GNP on turmeric leaves at regular intervals. Application of β-D-glucan nanoparticles enhanced the level of defense related enzymes in leaves and rhizomes, which correlated well with new isoforms of the enzymes. Qualitative differences in isoforms of these defense enzymes were investigated during the hereby time-course study. In general, the expression of chitinase activity was comparatively lower in rhizomes than in leaves. Chitinase and chitosanase activity reached maximum values during the 7th month. Exploiting the nanoparticle (derived from natural polysaccharide) potential may refer to induce defense enzymes that may diminish the use of toxic chemicals for disease control. Thus the use of nanoparticles could be proposed as an alternative, non-conventional and ecologically friendly approach for plant protection and hence for sustainable agriculture.

Keywords: Curcuma, defense enzyme, disease control, enzyme, nanotechnology

Introduction

Nanotechnology is gaining significant promise in agricultural sector for increased protection (Baruah and Dutta, 2009; Nair *et al.,* 2010; Ghormade *et al.,* 2011). Nanoparticles prepared from biopolymers or natural sources possess advantages such as availability from replenishable agricultural resources, biocompatibility, biodegradability and ecological safety (Ghormade *et al.,* 2011). Reports show that β-glucans play an important role during pathogen infection by inducing systemic resistance in plants (Yamaguchi *et al.,* 2000; Sriram *et al.,* 2003; Shinya *et al.,* 2006; Shetty *et al.,* 2009). However, the size of the glucan limits its wide spectrum application, since plant cell wall acts as a barrier for easy entry of any external agent.

Higher plants have the ability to initiate various defense mechanisms when they are exposed to biotic and abiotic stimuli. Chitinases and chitosanases are hydrolytic enzymes induced during biotic and abiotic stress (Mauch *et al.,* 1988; Grenier and Asselin, 1990; Graham and Sticklens, 1994). Also, the substrates chitin, chitosan and β-1,3 glucan are the major cell wall components of phytopathogenic fungi.

Hence, this study aimed to evaluate the potential of β-D-glucan nanoparticles for induction of chitinases and chitosanases in turmeric plants; the experiment developed under glass house condition.

Materials and Methods

Rhizomes of *Curcuma longa* (syn. *C. domestica*) cultivar 'Erode local' (susceptible) were obtained from farmer's field at Erode, Tamil Nadu, India.

GNP preparation and foliar application of the nanoparticles

GNP was prepared as described by well established protocol (Anusuya and Sathiyabama, 2014). Rhizomes were thoroughly washed with running tap water thrice, followed by glass distilled water; surface was sterilized by immersion in sodium hypochlorite 0.001% (v/v) solution for 15 minutes, followed by several rinses with sterile distilled water. The rhizomes (2-3 rhizomes, each with 3 nodes) were planted in earthen pots (27 cm diameter, 26 cm height) containing soil and manure, being maintained under glass house condition. GNP (0.1%, w/v) were applied to 30 day old plants (5 ml/plant) by foliar spray method, at a regular interval of 30 days, up to 210 days, as reported earlier (Anusuya and Sathiyabama, 2015). Water sprayed plants served as control. The leaves were removed after 210 days and the rhizomes were left for another 30 days before harvest. For each experiment, 30 plants were used and replicated thrice.

Protein extraction and enzyme assays

Leaves and rhizomes were collected from control and treated turmeric plants at regular intervals and used for extraction. The samples (1 g / 2 ml) were homogenized with potassium phosphate buffer (0.02 M, pH 7.6) and centrifuged for 10 min at 4 °C. The clear supernatant was used as source of protein, for enzymes.

Chitinase (EC 3.2.1.14) activity was assayed by the method of Reissig *et al*. (1955) using colloidal chitin as substrate. N-acetylglucosamine was used as standard. One

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unit of chitinase was defined as the amount of enzyme that liberated 1 µM of N-acetylglucosamine per minute under assay condition.

Chitosanase activity (EC 3.2.1.132) was determined by measuring the reducing sugars produced from chitosan. These sugars were estimated by Nelson (1944) and Somogyi (1952), using chitosamine HCl as standard. One unit of chitosanase was defined as the amount of enzyme that liberated 1 µM of reducing sugar, as chitosamine, per minute, under assay condition.

Gel electrophoresis

Protein samples (40 µg) from leaves and rhizomes of control and treated plants were separated on SDS-PAGE (10% separation gel) by the method of Laemmli (1970) along with standard marker protein from Bio Rad Chem Co USA. For chitinase localization, 0.1% (w/v) glycol chitin was included in the separation gel. After electrophoresis, the gel was stained with calcofluor white M2R according to the procedure of Trudel and Asselin (1989). Chitosanase localization was carried out according to the method of Grenier and Asselin (1990) whereas the separation gel contained 0.02% glycol chitosan. After electrophoresis, the gel was stained with 0.02% (w/v) coomassie brilliant blue.

Statistical analysis

All data were subjected to one-way analysis of variance to determine the significance of individual differences at $p < 0.01$ and 0.05 levels. All statistical analysis was conducted using SPSS 16 software support.

Results and Discussion

In the present study, it was examined the effect of GNP treatment and the ability to trigger induction of defence enzymes such as chitinase and chitosanase in turmeric plants. The basal level of chitinase activity was recorded in control and treated plants. Leaves of GNP treated plants showed significant increase in chitinase activity when compared to control. The maximum chitinase activity was observed in the 7th month (Fig. 1a). A similar pattern of increase in chitinase activity was observed in rhizomes of treated plants (Fig. 1a). The chitinase activity was higher in GNP treated plants, whereas control plants showed the least activity.

Significant difference was observed in the chitinase banding pattern among leaves of control and treated plants. Constitutive chitinase isoforms with molecular mass 60, 50, 36.8 and 19.3 kDa were induced in the 1st and 2nd month after GNP treatment. In the 2nd, 3rd and 4th month, a new isoform (77.8 kDa) was observed in GNP treated plants. In addition to this, 22 kDa chitinase isoform was observed in the 3rd, 4th and 5th month. In months 4, 6 and 7, 32 kDa isoform was observed in GNP treated plants. Apart from this, GNP treated plants showed a new isoform of molecular mass, respectively 34 kDa, in the 7th month (Fig. 1b).

In general, the expression of chitinase activity was comparatively lower in rhizomes than in leaves. In the 1st, 2nd and 3rd month, 34 kDa new chitinase isoform was observed in GNP treated plants. Another new chitinase isoform of molecular mass, 77.8 kDa, was observed in months 1, 5 and 8 respectively. Apart from this, GNP

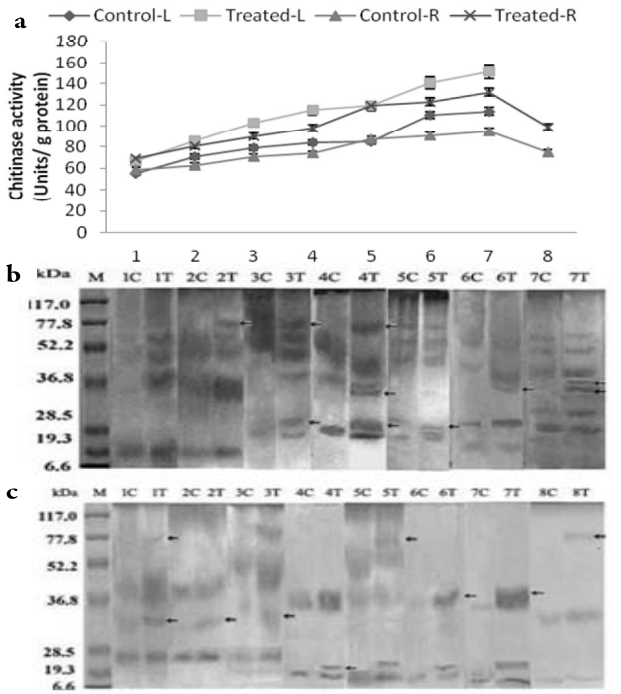

Fig. 1. Chitinase activity (a) in control and treated turmeric plants (L- Leaf; R- Rhizome); Localization of chitinase isoforms in leaves (b) and rhizomes (c) of turmeric plants on SDS-PAGE (10%)

M - Marker protein standard (Bio Rad Chem. Co., USA); C - Control; T - Treated; 1 to 8 - age of the plant in month. Molecular weight of marker protein: 117.0 kDa- β-Galactosidase; 77.8 kDa- BSA; 52.2 kDa- Ovalbumin; 36.8 kDa- Carbonic anhydrase; 28.5 kDa- Soybean trypsin inhibitor; 19.3 kDa- Lysozyme; 6.6 kDa- Aprotinin.

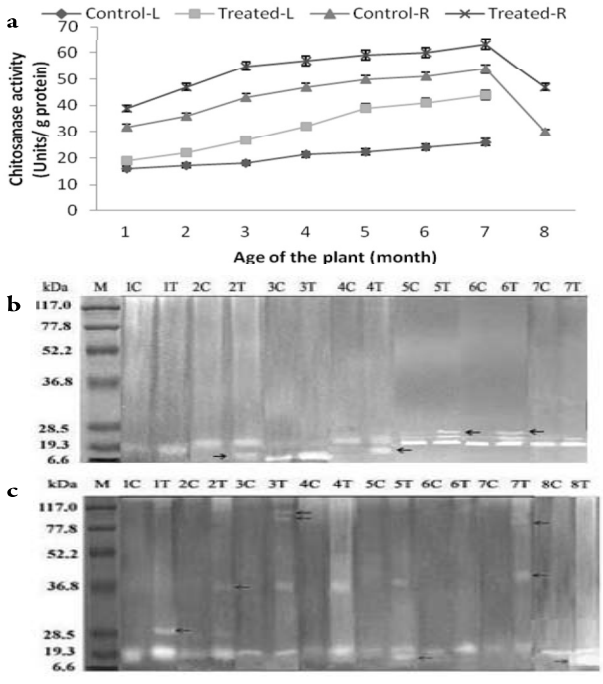

Fig. 2. Chitosanase activity (a) in control and treated turmeric plants (L- Leaf; R- Rhizome); Localization of chitosanase isoforms in leaves (a) and rhizomes (b) of turmeric on SDS-PAGE (10%)

M - Marker protein standard (Bio Rad Chem. Co., USA); C - Control; T - Treated; 1 to 7 - age of the plant in month. Molecular weight of marker protein: 117.0 kDa- β-Galactosidase; 77.8 kDa- BSA; 52.2 kDa- Ovalbumin; 36.8 kDa- Carbonic anhydrase; 28.5 kDa- Soybean trypsin inhibitor; 19.3 kDa- Lysozyme; 6.6 kDa- Aprotinin.

treated plants showed new chitinase 22 kDa isoform from 4th to 7th month. Induced isoform of molecular mass 36.8 kDa was observed in 6th and 7th month of GNP treated plants (Fig. 1c).

An increase in chitosanase activity was observed in treated plants compared to control. Chitosanase activity showed a gradual increase in both leaves and rhizomes of treated plants and reached its peak in the 7th month (Fig. 2a). The chitosanase activity was comparatively higher in rhizomes than in the leaves of turmeric plants.

As in Fig. 2b, 19.3 kDa chitosanase isoform was observed in the 1st month leaves of control and treated plants and remained up to the 7th month. GNP treated plants revealed a new chitosanase isoform of molecular mass 6.6 kDa in the 2nd month. In the 4th month, another new constitutive chitosanase isoform of molecular mass 15 kDa was observed in GNP treated plants. During the 5th and 6th month, GNP treated plants showed new chitosanase isoform of 25 kDa. Induced chitosanase isoform of 28.5 kDa was observed in the 7th month (Fig. 2b).

Rhizomes of GNP treated plants revealed new chitosanase isoforms with molecular masses of 28.5 and 36.8 kDa in the 1st and 2nd month respectively. The 36.8 kDa isoform remains until the 5th month. In the 3rd month two new isoforms 95 and 110 kDa were observed. In addition, GNP treated plants showed a new isoform of 15 kDa in the 5th month. Two new chitosanase isoforms of molecular masses 90 and 48 kDa were observed in 7th month in GNP treated plants. Another new chitosanase isoform of molecular mass 8 kDa was observed in the 8th month (Fig. 2c).

In the present study, it had been evaluated the effect of foliar application of β-D-glucan nanoparticles on defense enzymes' induction. Plant cell wall normally acts as a barrier for easy entry of any external agents, only nanoparticles, smaller than the pore diameter of the cell wall, can easily pass through and reach the plasma membrane (Moore, 2006; Navarro *et al.,* 2008). They may also cross the membrane using embedded transport carrier proteins or through ion channels. In the cytoplasm, the nanoparticles may bind with different cytoplasmic organelles and interfere with the metabolic processes at that site (Jia *et al.,* 2005). When nanoparticles are applied on leaf surfaces, they also enter through the stomatal openings or through the bases of trichomes and then translocated to various tissues (Fernandez and Eichert, 2009; Uzu *et al.,* 2010; Anusuya and Sathiyabama, 2015).

The interaction of plant cell with the nanoparticles resulted in modification of plant gene expression and associated biological pathways were reported (Nair *et al.,* 2010). Chitinase and chitosanase play a role in defense against invading pathogens because of their potential to hydrolyze fungal cell wall polysaccharides (Pozo *et al.,* 1998).

Conclusions

Application of β-D-glucan nanoparticles enhanced the level of defense related enzymes such as chitinases and chitosanases in leaves and rhizomes, which correlated well with new isoforms of the enzymes. Results of the hereby study suggested that the β-D-glucan nanoparticles application showed significant impact on induction of defense related enzymes, such as chitinases and chitosanases, under glass house condition. However, further work is necessary to identify the genes which undergo up regulation due to application of β-D-glucan nanoparticles to turmeric plants. Nanoparticle based formulations required in low volume with high value application makes the control more acceptable than conventional pesticides, which have negative environmental impact. Hence, bio-based nanoparticle formulations could have great potential as novel agrochemicals with high specificity and improved functions.

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