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TEMPERATURE MEDIATED CHITINASE PRODUCTION DURING RHIZOCTONIA ROOT AND CROWN ROT DISEASE IN SUGAR BEET (*BETA VULGARIS* L.) TAP ROOTS

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TEMPERATURE MEDIATED CHITINASE PRODUCTION DURING RHIZOCTONIA ROOT AND CROWN ROT DISEASE IN SUGAR BEET (*BETA VULGARIS* L.) TAP ROOTS.

By

Subashini Nagendran

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ABSTRACT

TEMPERATURE MEDIATED CHITINASE PRODUCTION DURING RHIZOCTONIA ROOT AND CROWN ROT DISEASE IN SUGAR BEET (*BETA VULGARIS* L.) TAP ROOTS.

By

Subashini Nagendran

Rhizoctonia root and crown rot is a serious disease of sugar beets in the US. Warm weather of mid-summer favors the development of rot but by mid-August, the rotting of tissue ceases and the infected tissue is restricted and demarked from healthy tissues. Sugar beet leaves produce chitinases as pathogenesis-related (PR) proteins in response to infection by *Cercospora beticola*. Experiments were carried out to determine if the temperature-mediated defense response of sugar beet tap roots to Rhizoctonia root and crown rot involves induction of chitinase isozymes in a moderately susceptible sugar beet cultivar. Native polyacrylamide gel electrophoresis (PAGE) analysis was employed to study the pattern of temperature-mediated chitinase induction over time in control (water), *R. solani*, and chitosan (abiotic inducer) treatments at two different incubation temperatures 10°C and 28°C. Our study suggest that induced production of chitinases during defense is a generalized wound response.

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CHAPTER ONE

DESCRIPTION OF THE CROP, PATHOGEN AND DISEASE:

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DISEASE --RHIZOCTONIA ROOT AND CROWN ROT.

CROP : SUGAR BEET (*BETA VULGARIS* L.)

The sugar beet (*Beta vulgaris* L.) belongs to the family Chenopodiaceae. Economically important species in this family include sugar beet, fodder beet/mangolds, red table beet, Swiss chard/leaf beet and spinach (*Spinacia oleracea*). Sugar beet is normally a biennial species. However, under certain conditions, it can act as an annual (Smith, 1987). The sugar beet plant develops a large succulent tap root in the first year and a seed stalk the second year. For seed production, however, an over-wintering period of cold temperatures of 4 - 7°C (vernalization) is required for the root to bolt in the next growing season and for the reproductive stage to be initiated (Smith, 1987). Sugar beet roots are processed into white sugar for food, as well as pulp and molasses for feed or industrial applications. Sugar beets are rarely used as a raw commodity. Sugar beet tap roots contain about 18% of sucrose per fresh weight and up to 75% of sucrose per dry weight (Elliott and Weston1993). Sugar is a multi-purpose carbohydrate that contributes significantly to the flavor, aroma, texture, color and body of a variety of foods. In addition to processing pure sugar, sugar factories also produce a by-product known as dried sugar beet pulp. This pulp is used as feed for cattle and sheep. Another important by-product is sugar beet molasses, a viscous liquid containing about 48% sucrose, which cannot be economically crystallizEditted by Sugar beet molasses is used for production of yeast, chemicals, pharmaceuticals, as well as in the production of mixed cattle feeds.

Currently, sugar beet is the major sugar crop grown in temperate regions of the world (Winner 1993).

Varieties of *Beta vulgaris* L. were developed through selective cultivation of Sea beet, *Beta maritime,* that is indigenous to the Mediterranean and the Atlantic seaboard of Europe as far north as the Baltic. Sea beet does not have a swollen root, like most of the varieties that have been derived from it. The first references to beet which can be dated accurately, occur in two comedies written by Aristophanes around 420 BC (Winner 1993). Table beet was eaten in Roman times at which time it was a long, white root. The swollen red root originated in about the mid 1500's. Its color is the result of high concentrations of red betalains. Fodder beet contains large white or yellow swollen roots developed in the 1700's for feeding livestock. By 1750 a process had been developed in Prussia for extracting sugar (sucrose) from sugar beet. During the Napoleonic wars British blockades cut off cane sugar supplies to the European continent and so the growing of beets for sugar became economical and was also encouraged by Napoleon and the King of Prussia. Through selection, the sucrose level in the beets eventually reached about 18%. By 1900, beet sugar production in Europe was nearly as great as World cane sugar production (Winner 1993). Early breeding techniques for sugar beet were developed by the USDA and include cytoplasmic male sterility, monogerm seeds and hybrid vigour (Panella, 1996). Cytoplasmic male sterility(CMC) is a maternally inherited condition involving a plant's inability to produce functional pollen. It is commonly found in natural plant populations and is most frequently caused by chimerical mitochondrial genes

(Schnable and Wise, 1998). Crosses between CMS mother lines and fertile pollinator lines being used to breed high quality sugar beet lines. The term used for propagule of sugar beet - the "seed", is technically a fruit. Naturally the sugar beets produce compound fruits which results in multigerm "seeds". Today, all U.S. sugar beet cultivars are monogerm hybrids. The use of monogerm sugar beet seed has greatly reduced the need to thin clusters of sugar beet seedlings, a high labor demanding requirement when multigerm seed was planted (Smith, 1987). Private seed companies now dominate sugar beet breeding concentrating on varieties which produce high sucrose concentrations, have disease and pest resistance as well as herbicide tolerance (http://www.sbreb.org/brochures).

PATHOGEN: *RHIZOCTONIA SOLANI*

Rhizoctonia solani Kühn. (AG2-2) [Teleomorph: *Thanatephorus cucumeris* (Frank) Donk] is a soil borne pathogen. This fungus has diverse mode of life such as saprophytic, pathogenic and mycorrhizal association with orchids (Andersen and Rasmussen 1996). There are presently about 120 species described within genus Rhizoctonia. Many are saprophytic while others cause economically important diseases on crop plants such as sugar beet, cereals, potato, vegetables, and fruit trees (Ogoshi, 1996). The current species concept stipulates that isolates of *R.solani* posses the following characteristics: a) some shade of brown hyphal pigmentation, b) brancing near the dital septum of cells in young vegetative hyphae, c)constriction of hyphae and formation of septa a short distance from the point of origin of hyphal branches, d)dolipore septa and e)multinucleate cells in young vegetative hyphae (Sneh, *et al* 1998). The species of *Rhizoctonia* consist of a diverse collection of teleomorphs belonging to different genera, namely Helicobasidium, Thanatephorus, Ceratobasium, Waitea, Tulasnella and Sebacina. Of the teleomorphs, Thanatephorus and Ceratobasium are most studied because several of these are pathogenic on important crop plants (Ogoshi, 1996). *R. solani* is a heterothallic, mycelia sterilia. Fungal inocula are hyphae and sclerotia. The pathogen does not switch to teleomorph stage, under normal laboratory cultural conditions. It does not often produce sexual spores under normal environmental conditions, but survives as resistant over-wintering structures called sclerotia and bulbils, which

are compacted masses of hyphae. Genetic relationships among strains are determined by their abilities to fuse in culture, and are called anastomosis groups (AG). Anastomosis in *R. solani* is defined as a manifestation of somatic compatibility between hyphae of different but related strains (Anderson 1982). Grouping *R.solani* by anastomosis reaction does not always correspond to grouping by host specificity, pathogenicity, colony morphology or other physical features. For example, the AG that causes disease on wheat is the same one that induces damping-off in sugar beet seedlings (AG-4), but is different from the one that causes Root and Crown rot in beets (AG2-2). Another disease of sugar beet called Dry Rot is caused by *R. solani*, but is caused by different field isolates than those causing Rhizoctonia root and crown rot. *R. solani* AGs 1, 4 and 5 also cause disease on sugar beet, but at much lower frequencies. (Naito et. al. 1997, Windels and Nabben 1989 , Rush et. al. 1994) It is reported that different AGs penetrate the host plant differently (Ogoshi, 1996) *R. solani* produces cutinases and it is suggested that this may in part be involved in tissue penetration (Kolattukudy, 1985). When the genetics of *R. solani* is considered there are two views, the non-sexual species view and sexual species view (Adams 1996). The apparent absence of the sexual fruiting in nature and the difficulty producing it in the laboratory favors the non-sexual species view. Heterokaryosis is considered as the mechanism that permits genetic recombination during meiosis under non-sexual species view. Sexual morphology and dispersal mechanism of sexual spore in *Rhizoctonia*-like organism is well developed and under very critical laboratory conditions

R.solani has been induced to produce sexual fruiting(Adams 1996). These observations support for the sexual species view to explain the genetics of this fungal species. To understand the diversity in the genetics of *Rhizoctonia* further research is needed on basidiospores, its survival, homokaryotic colonies, its competitiveness and sporulation.

DISEASE :RHIZOCTONIA ROOT AND CROWN ROT

R. Solani AG2-2 is the major group that causes sugar beet root and crown rot disease. Rhizoctonia root and crown rot is endemic and economically important in relatively humid regions of the USA. Average disease loss is estimated to be 2% of the crop (Schneider and Whitney 1986). Crown and root rot occurs on older plants, generally associated with canopy closure.

Sclerotia, undifferentiated aggregates of thick walled melanized hyphae, are important sources of inoculum and are the primary survival structures. The sclerotia are formed in soil and plant residues and survive for a long time (Sumner, D. R. 1996) They germinate and form mycelial threads that can grow toward the plant. The germination is moisture and temperature dependent and it is stimulated by plant exudates (Reddy 1980). This saprophytic growth stimulation will increase the inoculum density and may consequently influence disease formation. Once the hyphae come in contact with the plant, they grow over the plant surface. The hyphae becomes flattened and closely and firmly attaches to the plant surface (Armentrout *et al.* 1987). The infection process starts once the pathogen attaches itself on the host tissue. The presence of mucilaginous sheath around the attached hyphae is reported and this sheath is not present around the hyphae behind the point of attachment (Matsuura 1986). The attached hyphae start to follow the anti-clinal walls of the contiguous epidermal cells. About 15 hours after the initial contact side branches are formed at right angle to the main mycelium. They are called T-shaped branches

and are a determining characteristic for infection structures of *R. solani*. These braches either give rise to short swollen hyphae, or appressorial structure, or they repetitively branch and form complex infection structures called infection cushion. Swollen tips of these various structures may give rise to infection pegs. A peg will penetrate the cuticle and epidermal cell wall. *R. solani* primary invasion sites in sugar beet are the lower surface of petioles in contact with soil; natural cracks in the crown, lenticels on the taproot and lateral root development sites. Following penetration virulent strains of *R.solani* AG2-2 isolates progressively invade and colonize the vascular tissues of the sugar beet crowns and roots. These observations show how mode of penetration and subsequent tissue colonization play an important role in causing Rhizoctonia root and crown rot disease. The formation of the thin peg may allow the fungus to exert maximal hydrostatic pressure on the plant surface. Some hypo virulent strains are also melanin deficient suggesting that hydrostatic pressure may be important for penetration (Sneh, *et al.* 1989). Inside the plant, the primary hyphae rapidly invade the epidermal tissue and outer layer of the cortex, growing intracellularly as well as in the intercellular space depending on the strain (Yang *et al* 1992). In susceptible host tissue, colonization is rapid.

Early symptoms include sudden and permanent wilting of leaves and black necrosis of petioles at the crown. Wilted plants seldom recover, and after dying, leaves often form a dry, dark rosette. Infection begins as discrete, dark elliptical lesions on root surface. These lesions may grow together and eventually cover the entire root surface as disease progresses. Infections may also start in the

crown and move downward. Infected roots usually remain firm, and rot seldom penetrates into the interior of the root until advance stages. A clear margin is often seen between infected and healthy tissues, and extensively rotted roots will also exhibit cracks on the surface (Schneider and Whitney 1986). In the field one can observe long patches of dead and diseased plants.

The disease severity caused by *R. solani* is influenced by many factors including inoculum abundance, nutritional state of the pathogen and environmental conditions, primarily temperature, which influences the relative growth rates of both pathogen and host. Rhizoctonia disease decline (RDD) or soil suppressive-ness due to monoculture occurs in sugar beet Rhizoctonia root and crown rot in naturally infected fields with *R. solani* AG2-2 and *Bacillus* species were reported as possible agents for disease suppressiveness (Hyakumachi *et al.* 1990) .

The pathogen over winters in soil and plant debris as hyphal fragments and sclerotia, and becomes active when soil temperatures approach 25-33 °C. Rhizoctonia root disease may occur in almost any soil, but is most severe in heavy, poorly drained soils or in depressions in fields where water pools. Infection also commonly results when cultivation deposits soil into beet crowns (Schneider and Whitney 1986).

One of the most economical methods of disease management is planting of resistant cultivars locally adapted to the production area. Seed treatments with various fungicides will control damping-off of seedlings. Cultural practices that introduce soil into the crowns should be minimizEditted by Crop rotation with

small grains reduces number of pathogen survival structures in soil. Controlling susceptible plants like pigweed and maintaining field sanitation will also reduce disease incidence in beets (Schneider and Whitney 1986). Use of mixtures of resistant and susceptible sugar beet varieties decreases yield losses from Rhizoctonia root and crown rot disease (Johnson, D.J and Halloin, J.M 2000). A new fungicide Quadris, (azoxystrobin) is now available that shows promise for protection from the crown rot phase of the disease (Perr communication Halloin, J M. and Johnson, D.J. USDA-ARS, East Lanisng, MI.). Host resistance to *R. solani* is typically an incremental reduction in disease severity.

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CHAPTER TWO

TEMPERATURE MEDIATED CHITINASE PRODUCTION DURING RHIZOCTONIA ROOT AND CROWN ROT DISEASE IN SUGAR BEET (*BETA VULGARIS* L.) TAP ROOTS.

INTRODUCTION

Plants use various defense mechanisms to protect their tissues from pathogen infection. One response to pathogen attack involves synthesis of proteins with anti-microbial activity, such as chitinases and β 1-3 glucanases (Hammond and Jones 2000). Plant endo-chitinases (EC3.2.1.14) are extensively studied to correlate anti-fungal activity to plant defense mechanisms. Endo-chitinase from barley has been heterologously expressed in *E.coli* and its anti-fungal activity toward *Trichoderma reesei* was demonstrated (Andersen *et al.* 1997). The systematic name for endo-chitinase is poly[1,4-(N-acetyl-beta-D-glucosaminide)] glycanohydrolase; synonyms are 1,4-beta-poly-N-acetylglucosaminidase , chitodextrinase-N, chitotriosidase ,PLC-A , PLC-B and poly-beta-glucosaminidase. Chitinase catalyzes random hydrolysis of N-acetyl-beta-D -glucosaminide 1,4-beta-linkages in chitin and chitodextrins (Otha *et al.* 1995). Chitin, a linear homo-polymer of ß-1,4 linked N-acetylglucosamine, is a major component of fungus cell walls (Cabib and Sburlati 1988). Boller *et al.* (1983) reported that chitinases purified from ethylene-treated bean leaves exhibit lysozyme activity with the bacteria *Micrococcus lysodeikticus*. The enzyme has been isolated from several species of animals, plants, fungi and bacteria. Species of plants that have been demonstrated to produce chitinase include *Beta vulgaries, Oryza sativa* (Nishizawa *et al.* 1993) *Triticum aestivum* (Molano et al. 1979), *Glycine max* (Zikakis and Castle 1988), *Lycopersicon esculentum* (Pegg 1988), *Phaseolus vulgaris* (Boller *et al.* 1983), *Dioscorea opposite* (Tsukamoto

*et. al.*1984), *Arabidopsis thaliana* (Verburg and Huynh 1995), *Hordeum vulgare* (Andersen *et. al* 1997), *Brassica oleracea* (Chang *et. al.* 1996), *Cucubita maxima* (Kim *et. al.* 1999), and *Daucus carota* (*Kragh et. al.* 1996).

Generally, plant endo-chitinases are proteins with molecular weights of 25-35 kDa, and occur as monomers. They typically have broad pH optima around pH6 and are stable at temperatures up to 50°C (Boller *et al.* 1983). In addition to their postulated roles in plant defense, glycosylated acidic chitinases may have an important function in early plant somatic development. De jong *et al.* (1992) reported that addition of the 32 kD endo-chitinase to temperature-sensitive embryo cultures at a temperature non-permissive of embryo development, appeared to promote the formation of a correctly formed embryo protoderm. De los Reyes (1999) reported that chitinase genes are involved in mechanisms that protect the meristem from freeze-induced dehydration in bermudagrass.

Originally three classes of chitinases have been proposed based on their amino acid sequences (Shinshi *et al.* 1990). Chitinases in class I contain two domains; (i) the N-terminal chitin binding domain (ii) the chitinase domain, involved in catalytic activity. These two domains are linked through a short glycine/proline-rich region. Class II chitinases contain only the chitinase domain. Class III chitinases contain catalytic domain but show no sequence similarity to chitinases of class I and II. Collinge *et al.* (1993) proposed chitinase of class IV to include basic sugar beet chitinase IV, the basic rape chitinase ChB4 and acidic bean PR4 chitinase . These chitinases contain a cysteine-rich domain and a

conserved structure similar to class I but much smaller than class I due to four deletions. Chtinases were further classified as glycosyl hydrolases, as pathogenesis –related proteins and gene families.

Chitin is a major cell wall component of all the organisms of kingdom fungi, and the exoskeleton of arthropods, phyla that include many plant pests (Cabib and Sburlati 1988. Even though higher plants synthesize chitinases, no endogenous substrate for chitinase is reported in higher plants. Thus, it is speculated that plants' chitinases protect them from pest attack by lysing the insect exoskeleton or pathogen cell wall component chitin. It was observed that purified bean chitinase inhibits the growth of the fungus *Trichoderma viride* hyphal tips *in vitro* (Schlumbaum *et al.*1986). An acidic class III chitinase was found to accumulate in leaves of sugar beet during infection with *Cercospora beticola* (Nielson *et al.* 1993). Enhancing the endogenous production of chitinases in tobacco plants resulted in greater resistance to infection by fungal pathogens such as *Rhizoctonia solani* (Broglie *et al.* 1991; Vierheilig *et al.* 1993). These reports suggest a role for chitinases in the defense of plants against pathogenic fungi. Thus, it may be possible to genetically engineer resistance to certain fungi by regulating the expression of chitinases in host plants.

Sugar beet root and crown rot caused by *Rhizoctonia solani* and root storage rot caused by *Aspergillus* are more severe at warm (> 20 ° C) than at cool (< 20 ° C) temperatures(Schneider and Whitney 1986;Halloin and Roberts 1995). Halloin (1994) demonstrated that warm temperatures (> 20 ° C) are

conducive to *R.solani* infection of sugar beet tap roots, whereas cool temperatures (< 20 ° C) favor expression of host resistance that is accompanied by production of phenolic compounds (phytoalexins) at the infection boundary.

Ruppel (1986) reported that leaf spot caused by *Cercospora beticola* is favored in warm temperatures. In contrast to these observations, seedling disease caused by *Pythium ultimum* and *Phoma betae* are more severe in cool soils (Leach, 1986). Disease is an outcome of interaction among host, pathogen and the environment and the effect of temperature on these components should be critically analyzed to study temperature-mediated resistance. Field observations have shown that temperature is a mitigating factor in sugar beet diseases caused by fungi. Warm temperatures favor disease development while cool temperatures suppress disease severity. In field-infected sugar beet tap roots the progress of Rhizoctonia root and crown rot is controlled under low temperature conditions and a well demarked area of diseased tissue and healthy tissue can be seen .

Experiments were carried out to determine if the temperature-mediated defense response of sugar beet tap roots to Rhizoctonia root and crown rot involves induction of chitinase isozymes in a moderately susceptible sugar beet cultivar. Native polyacrylamide gel electrophoresis (PAGE) analysis was employed to study the pattern of temperature-mediated chitinase induction over time in control (water), *R. solani*, and chitosan (abiotic inducer) treatments at two different incubation temperatures 10°C and 28°C.

MATERIALS AND METHODS

Plant material

Mature healthy taproots of sugar beet (*Beta vulgaris* L., cultivar Hilleshög Mono Hy E17) were produced in field plots at East Lansing MI (MSU Botany and Plant Pathology farm).

Biotic inducer (fungal inocula)- *Rhizoctonia solani* Kuhn AG 2-2 grown on millet seeds.

The fungus *Rhizoctonia solani* AG 2-2 was grown on corn meal agar (CMA) in petri dishes at room temperature. De-hulled seeds of millet that were sterilized three consecutive days at 120°C for 20 minutes, were placed as a single layer on the actively growing 3-day-old CMA fungal culture and were incubated at room temperature under normal laboratory illumination on the bench top for another 4 days. The millet was completely colonized with the fungus and was used as the inoculum for experiments with sugar beet tissues.

Abiotic inducer - Chitosan (1mg/ml)

Chitosan from crab shells was obtained from Sigma-Aldrich (Cat. No. C-3646). Stock solutions of chitosan (1 mg/ml) were prepared in 10 mM acetic acid (pH-adjusted to 5.3±0.1 with NaOH) (Hadwiger and Beckman 1980).

Treatment

Healthy sugar beet tap roots weighing 2 - 3 kg were harvested at East Lansing, MI, and rinsed free of soil. Roots were cut into 2 cm (wide) X 2 cm (deep) X 10cm (long) pieces. Three holes, 3mm in diameter X 1 cm deep were drilled into each piece with approximately 3.5 cm separation between holes, and these holes were filled with water, chitosan solution (1mg/ml) or *Rhizoctonia* inoculum (*R. solani* grown on millet caryopses). Water and chitosan solutions were absorbed by the tissue pieces within 30 minutes. Treated tissue pieces were placed within perforated plastic bags, and incubated at either 10°C or 28°C for 0, 12, 24, 36, 48, 72, 96, 120 or 144 hours. Tissue samples were collected by boring sections (7mm diameter X 10mm deep) within the tissue pieces, thus providing 10mm long X 7mm diameter cylinder of tissue. Each tissue block contained all three treatments (water, chitosan solution (1mg/ml) or *Rhizoctonia* inoculum) separately , and all combinations of treatments and temperatures were done in triplicates, with each of the triplicate replications being done on tissues from three different sugar beet roots. A diagrammatic representation of the tissue pieces and sample collection is shown in Plate 1. Harvested samples were stored at -20°C, and were subsequently ground to powder under liquid nitrogen with a mortar and pestle prior to enzyme preparation.

Plate 1: Inoculation block

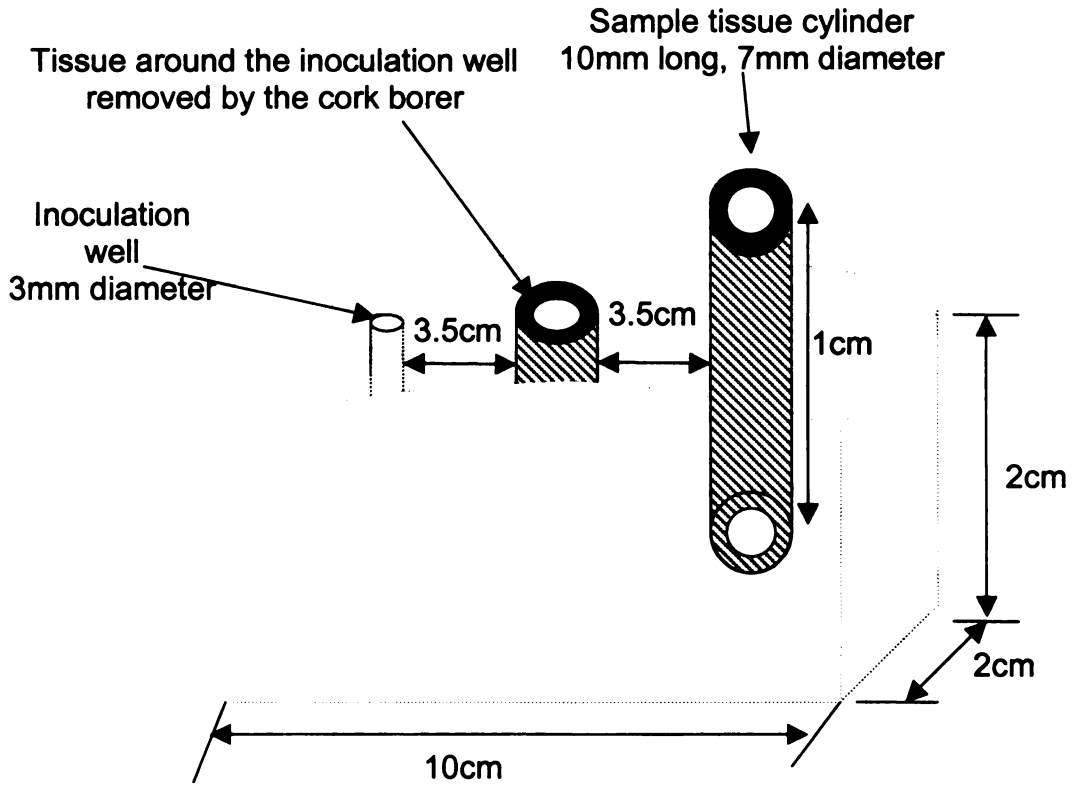

Plate 1: Inoculation block: Model system of sugar beet tap root blocks inoculated in the laboratory with water (control), *Rhizoctonia* inocula in millet seeds or abiotic inducer chitosan. Each tissue block had 3 inoculation wells about 3.5 cm apart. Not drawn by scale.

Extraction of protein

Homogenization buffer (0.1M Sodium Phosphate buffer (pH 6.0), 15mM 2-β Mercaptoethanol) was added to the sample in a ratio of 3:1(V/W) and the tissue was ground in an ice bath and centrifuged at 10,000 rpm for 20 min. The clear supernatant preparation (crude protein extract) was stored at–80^{0}C until use.

Measurement of Total protein

The Bradford dye-binding procedure (Bradford 1976), a simple colorimetric assay for measuring total protein concentration, was used to measure the total protein concentration of preparations. The protein assay is based on the color change of Coomassie Brilliant Blue G-250 dye in response to various concentrations of protein. The dye binds to primarily basic (especially arginine) and aromatic amino acid residues. A standard curve was created for use with each set of experiment samples. Bovine serum albumen (BSA) (Bio-Rad catalog # 500-0007) was used in concentrations of 0.0, 0.2, 0.6, 0.9, 1.2, 1.5 mg/ml. Each 10 μl of BSA solution was mixed with 2.0 ml of Bradford solution (20% V/V Bradford reagent [Bio-Rad catalog # 500-0006]: H_2O). The corresponding absorbance was measured with a diode array spectrophotometer at 595nm. The concentrations were plotted against the absorbance readings.

Each 10 μl of sample protein extract was mixed with 2.0 ml of 20% Bradford Solution and absorbance readings were taken immediately, and were converted to concentration using the standard curve.

Polyacrylamide gel electrophoresis (PAGE) under native condition

PAGE was performed preserving the chitinases enzyme activity under native condition at pH 8.9 according to the method of Davis (1964) using 10 % (W/V) polyacrylamide resolving gel (8 cm height, 8 cm width and 1.5 mm thickness) and 10% stacking gel 1 cm (H). The gels contained 1 % (V/V) of 1 % (W/V) glycol chitin (modified according to Trudel *et al.* 1989). For each protein sample, pipetted a volume of crude protein extract that contained 6 μg of total protein was mixed with 8 μl of loading buffer (1.0 ml of electrophoresis buffer, 3.0 ml of glycerol, 0.2 ml of 0.5% bromophenol blue, 5.8 ml of deionized water and loaded into Polyacrylamide gel well. Bovine serum albumin (BSA) (4 μg) was included as a control protein with no chitinolytic activity. Electrophoresis was done at room temperature for about 4 hours at 45 V with 2 buffer systems (McLellan, T., 1982): upper buffer pH 8.91 at 25 $^{\circ}$ C and lower buffer pH 8.07 at 25° C. The gel was cut to separate the control proteins that was loaded with BSA, from the samples after electrophoresis. The control protein gel that was loaded with BSA was stained with Coomassie blue R-250. (0.25% m/v Coomassie brilliant blue R250, 45.4% v/v methanol, 9.2% v/v glacial acetic acid and 45.15% v/v dd H_2O (Modified from Payne 1976) The control protein gel sections were then de-stained over night at room temperature in a solution that contained 5% v/v

methanol, 7.5% v/v glacial acetic acid and 87.5% v/v dd H_2O. Gels were photographed under visible light.

Detection of chitinase activity after PAGE under native conditions.

Gels were incubated in 100 mM Sodium acetate pH 5.0 for 4 hours at 37 ^{0}C. They were rinsed once for about a minute with double distilled H_2O and incubated in 500mM Tris-HCl pH 8.9, 0.01% (M/V) calcoflour white) for 5 minutes in the dark at room temperature (Modified from Trudel *et al* 1989). The polyacrylamide gel contains the substrate glycol chitin, which will be hydrolyzed by chitinase enzyme that was separated during electrophoresis. Calcoflour white was bound only to un-hydrolyzed glycol chitin. Hydrolyzed glycol chitin gave banding pattern under UV light indicating the presence of chitinase enzyme at that protein band position. Gels were photographed under UV light using Eagle Eye II system (Strategene Ltd.).

RESULTS

The pattern of chitinase induction in the control (water), *R.solani* and chitosan (abiotic inducer) treatments was studied in moderately susceptible sugar beet (Cultivars E17) tap roots at two different incubation temperatures 10° C and 28° C, to ascertain whether host chitinases are possibly involved in temperature mediated Rhizoctonia crown and root rot disease resistance. Discoloration of tissues, indicative of either oxidation or rot was observed only in the treated tissues inoculated with *R.solani* and incubated at 28° C; These tissues showed progressive discoloration and decomposition of tissues starting at 36 hours after inoculation.

Even though equivalent total proteins were loaded to PAGE across all treatments (0 to 144 hours incubation), the tissues collected immediately after inoculation with the water (control), chitosan and *R.solani* (0 hours) all had low chitinase activity with two detectable chitinase bands (Figure 2.1). After an incubation time of 12 hours or more the chitinase activity increased and the number of bands in the gels resolved also increased. (Figure 2.2 through Figure 2.9). The effects of the interacting factors, host challenge (by chitosan or *R.solani*) temperatures (10°C or 28°C), and time (0 to 144 hours) on host production of chitinase are demonstrated in Figures 2.2 through 2. 9. The overall results of these interactions are summarized in Figure 2.10. The chitinase isozyme banding patterns on the polyacrylamide gels (figure 2.1 thru figure 2.9) are summarized in Table 2.1.

Table 2.1: Summary of chitinase isozyme migration pattern on polyacrylamide gel. Band named as 1,2,3 &4 refers to the bands that are labeled in figure 2.1 through 2.9.

Hours incub.	tempt. C	Treatment	# of ban-ds	Comments
0		Control	1	A very low intense band 2 is present in Chitosan and Rhizoctonia
0		Chitosan	2	treated tissue; Smearing is present; Relative mobility of band 1 &2 are
0		Rhizoc	2	between BSA 201kDa and 134 kDa
12	10C	Control	2	Band 1 & 2 are in high intense & have migrated more than
12	10C	Chitosan	2	0 hour treatments bands.relative mobility of band 1 between
12	10C	Rhizoc	2	28C Chitosan & Rhizoctonia treatments have higher intense
12	28C	Control	2	chitinase isozyme bands & smearing than 28C Control &
12	28C	Chitosan	2	10C Control, Chitosan and Rhizoctonia treatments.
12	28C	Rhizoc	2	
24	10C	Control	2	Band 1 & 2 are high intense & have migrated more than 0 hour treatments.
24	10C	Chitosan	2	bands are parallel to BSA 134 kDa; 10C Chitosan & 28C
24	10C	Rhizoc	2	Chitosan & Rhizoc treatments have stronger chitinase bands
24	28C	Control	2	& smearing than 28C Control & 10C Control & Rhizoctonia treatments.
24	28C	Chitosan	2	
24	28C	Rhizoc	2	
36	10C	Control	3	Compare to 10C Control other treatments -10C Chitosan Rhizoctonia
36	10C	Chitosan	3	& 28 C Control, Chitosan & Rhizoctonia, have stronger chitinase
36	10C	Rhizoc	3	isozyme bands &have migrated differently. 10C band 1 is parallel to
36	28C	Control	3	BSA 201kDaband 2 bet. 201 kDa & 134 kDa, band 3 parallel to 134 kDa.
36	28C	Chitosan	3	other treatments(10 & 28C Chitosan and Rhizoctonia & 28C Control)
36	28C	Rhizoc	3	band 1 between BSA 201kDa & 134kDa ,band 2 parallel to BSA 134kDa:
				band3 between BSA 134kDa & 67kDa close to 134kDa.

Table 2.1: Summary of chitinase isozyme migration pattern on polyacrylamide gel continued.

Hours incub.	tempt. C	Treatment	# of Band	Comments
48	10C	Rhizoc	3	Banding pattern similar to 36 hours
48	28C	Control	3	28C Rhizoctonia has smearing.
48	28C	Chitosan	3	
48	28C	Rhizoc	3	
72	10C	Chitosan	3	Migration of isozyme bands are comparble to that of 36 &48 hour
72	10C	Rhizoc	3	treatments.
72	28C	Control	3	
72	28C	Chitosan	3	
72	28C	Rhizoc	3	
96	10C	Control	3	Band 3 is weak & at different levels of migration -10C Control,Chitosan
96	10C	Chitosan	3	Rhizoc & 28C Rhizoc band3 migration is parallel to BSA 134kDa.
96	10C	Rhizoc	3	28C Control &Chitosan band 3 migrated bet. 134kDa 7 67kDa.
96	28C	Control	3	All treatments have smearing effect.
96	28C	Chitosan	3	
96	28C	Rhizoc	3	
120	10C	Control	3	10C Control, Chitosan & Rhizoctonia band 1 parallel to BSA 201kDa
120	10C	Chitosan	3	band 2 between BSA 201kDa & 134kDa, band 3 at BSA 134kDa.
120	10C	Rhizoc	3	28C Chitosan and Rhizoctonia band 1 parallel to BSA 201kDa ,band 2
120	28C	Control	2	parallel to BSA 134kDa & band 3 between BSA 134kDa & 67kDa.
120	28C	Chitosan		Smearing is present in 28C Chitosan and Rhizoctonia.
120	28C	Rhizoc		
144	10C	Control	3	weak band 4 is present in 10C Chitosan,Rhizoc ,28C Rhizoc
144	10C	Chitosan	3	The distance migrated by bands are varying between treatments
144	10C	Rhizoc	4	High intensity of smearing is present in 10C Chitosan &28C Chitosan.
144	28C	Control	3	
144	28C	Chitosan	4	
144	28C	Rhizc	4	

Compared to 0 hour incubated tissue, increases in chitinase activity occured even in water treated controls. Since under native gel electrophoresis conditions, migration of proteins depend on size, shape and charge of the protein, we can not determine the sizes of these resolved band. At 0 hour incubation the control treatment has one band and *Rhizoctonia* and Chitosan treated tissue have two bands- a high intense band and a very low intense additional second band. This difference may be due to wounding. At 12 hour, 28°C *Rhizoctonia* and Chitosan treated tissues have high intense bands. Similar pattern is observed at 24 hours incubation. By 36 hours of incubation, a third isozyme band is produced by all the treatments and control tissue at both incubation temperature suggesting this is a generalized wound response. As the incubation time is increased to 72 hours,96 hours, 120 hours and 144 hours, 28 °C incubated *Rhizoctonia* and Chitosan treated tissues have high intense banding pattern and smearing. Overall, chitinase induction appeared to occur faster, and at greater magnitude at 28° C than at 10°C.

Figure 2.1:PAGE- 0 hour incubation

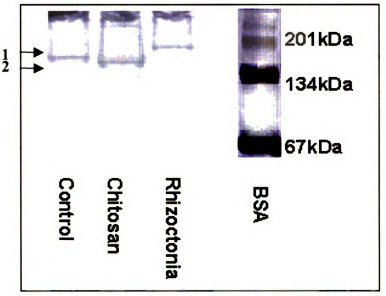

Figure 2.1: PAGE- 0 hours incubation : Native gel electrophoresis of proteins prepared from sugarbeet tap roots collected immediately (0 hours) after inoculation with water (control) Chitosan and *Rhizoctonia solani* AG2-2 and stained for chitinase hydrolysis. The chitinases bands are indicated by arrow and labeled as 1&2 from top. BSA is stained with Coomassie blue R-250.

Figure 2.2 : PAGE- 12 hours incubation

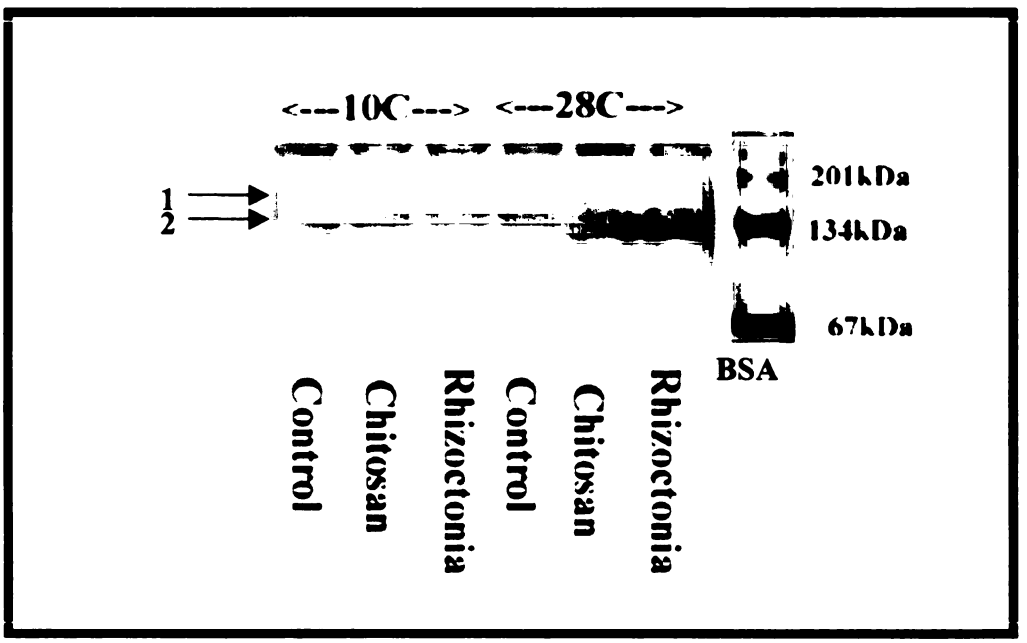

Figure 2.2 : PAGE- 12 hours incubation: Native gel electrophoresis of proteins prepared from sugarbeet tap roots collected 12 hours after inoculation with water (control) Chitosan and *Rhizoctonia solani* AG2-2 and stained for chitinase hydrolysis. The chitinases bands are indicated by arrow and labeled as 1&2 from top. BSA is stained with Coomassie blue R-250.

Figure 2.3 : PAGE- 24 hours incubation

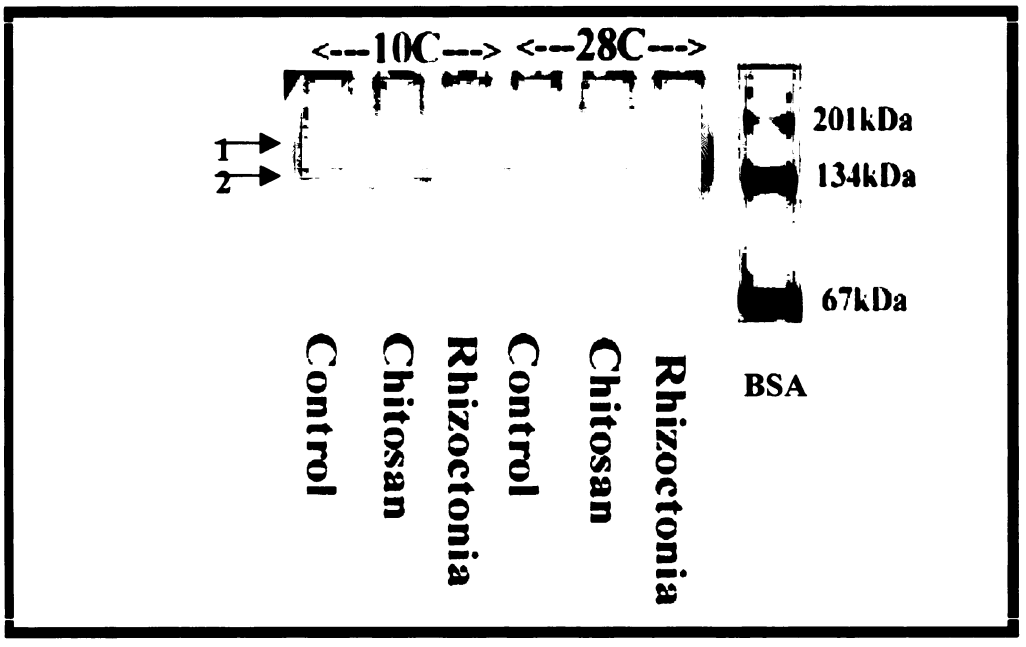

Figure 2.3: PAGE- 24 hours incubation: Native gel electrophoresis of proteins prepared from sugarbeet tap roots collected 24 hours after inoculation with water (control) Chitosan and *Rhizoctonia solani* AG2-2 and incubated at 10°C and 28°C and stained for chitinase hydrolysis. The chitinases bands are indicated by arrow and labeled as 1&2 from top. BSA is stained with Coomassie blue R-250.

Figure 2. 4 : PAGE- 36 hours incubation

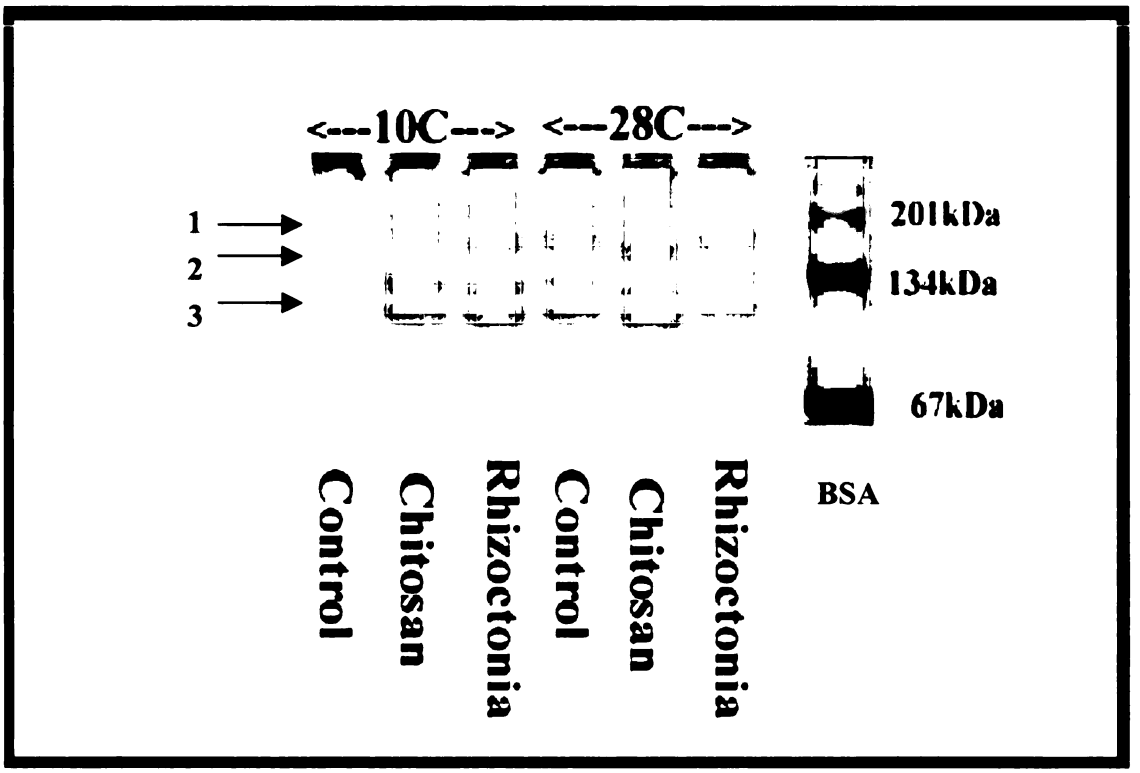

Figure 2.4: PAGE- 36 hours incubation: Native gel electrophoresis of proteins prepared from sugarbeet tap roots collected 36 hours after inoculation with water (control) Chitosan and *Rhizoctonia solani* AG2-2 and incubated at 10°C and 28°C and stained for chitinase hydrolysis. The chitinases bands are indicated by arrow and labeled as 1,2 & 3 from top. BSA is stained with Coomassie blue R-250

Figure 2. 5 : PAGE- 48 hours incubation

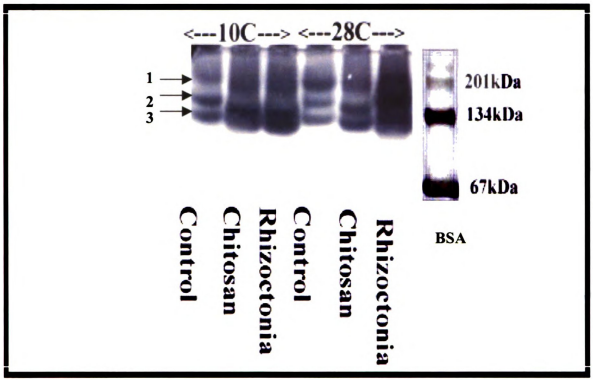

Figure 2.5 : PAGE- 48 hours incubation: Native gel electrophoresis of proteins prepared from sugarbeet tap roots collected 48 hours after inoculation with water (control) Chitosan and *Rhizoctonia solani* AG2-2 and incubated at 10°C and 28°C and incubated at stained for chitinase hydrolysis. The chitinases bands are indicated by arrow and labeled as 1,2&3 from top. BSA is stained with Coomassie blue R-250

Figure 2. 6: PAGE- 72 hours incubation

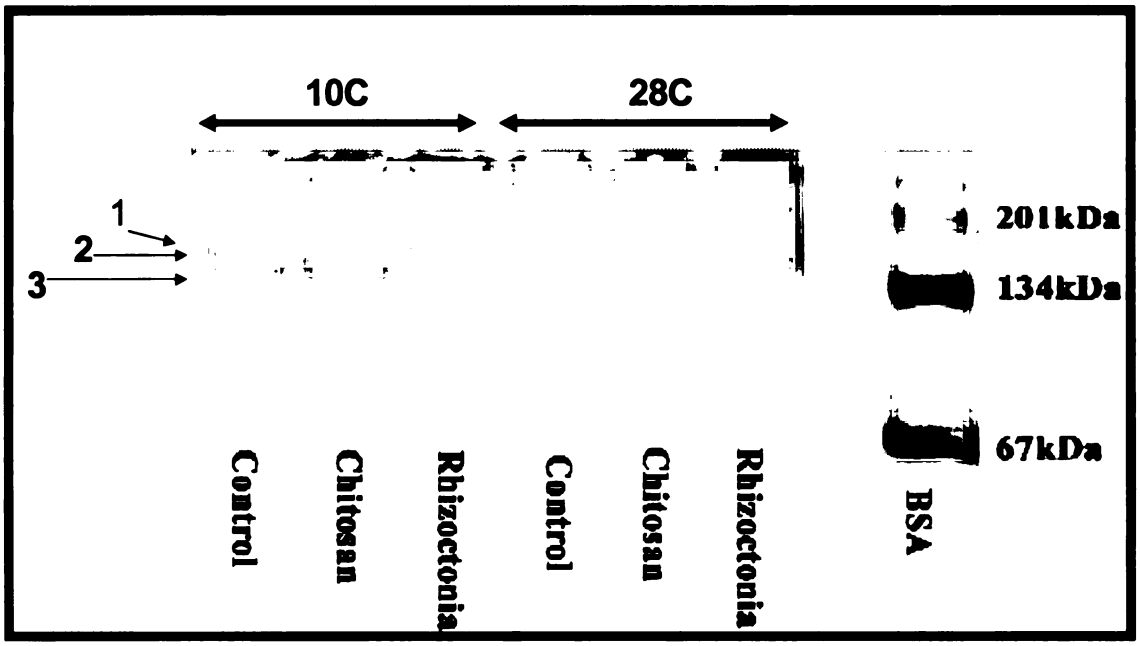

Figure 2.6: PAGE- 72 hours incubation: Native gel electrophoresis of proteins prepared from sugarbeet tap roots collected 72 hours after inoculation with water (control) Chitosan and *Rhizoctonia solani* AG2-2 and C and stained for chitinase and incubated at 10°C and 28°C hydrolysis. The chitinases bands are indicated by arrow and labeled as 1,2&3 from top. BSA is stained with Coomassie blue R-250

Figure 2. 7: PAGE- 96 hours incubation

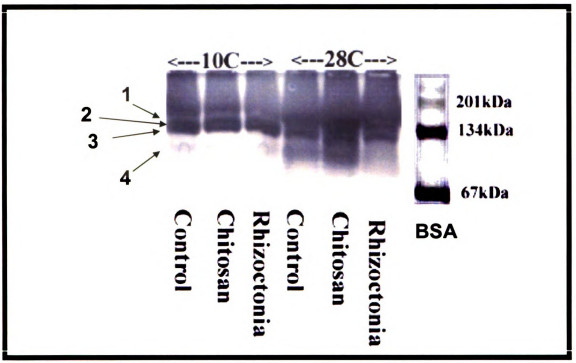

Figure 2.7: PAGE- 96 hours incubation: Native gel electrophoresis of proteins prepared from sugarbeet tap roots collected 96 hours after inoculation with water (control) Chitosan and *Rhizoctonia solani* AG2-2 and incubated at 10°C and 28°C and stained for chitinase hydrolysis. The chitinases bands are indicated by arrow and labeled as 1,2,3&4 from top. BSA is stained with Coomassie blue R-250

Figure 2. 8: PAGE- 120 hours incubation

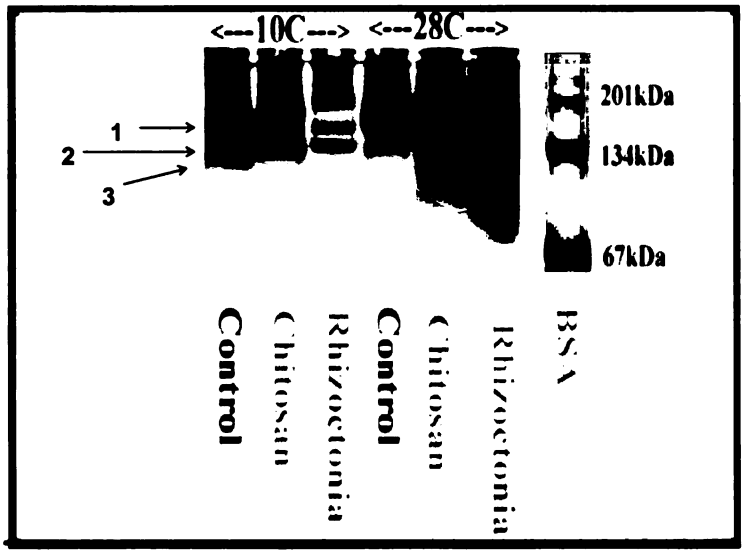

Figure 2.8: PAGE- 120 hours incubation: Native gel electrophoresis of proteins prepared from sugarbeet tap roots collected 120 hours after inoculation with water (control) Chitosan and *Rhizoctonia solani* AG2-2 and incubated at 10°C and 28°C and stained for chitinase hydrolysis. The chitinases bands are indicated by arrow and labeled as 1,2 &3 from top. BSA is stained with Coomassie blue R-250

Figure 2. 9: PAGE- 144 hours incubation

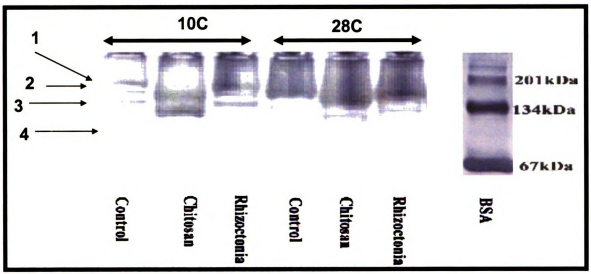

Figure 2.9: PAGE- 144 hours incubation: Native gel electrophoresis of proteins prepared from sugarbeet tap roots collected 144 hours after inoculation with water (control) Chitosan and *Rhizoctonia solani* AG2-2 and incubated at 10°C and 28°C and stained for chitinase hydrolysis. The chitinases bands are indicated by arrow and labeled as 1,2,3 & 4 from top. BSA is stained with Coomassie blue R-250

Figure 2.10: Incubation temperature Vs. Incubation time

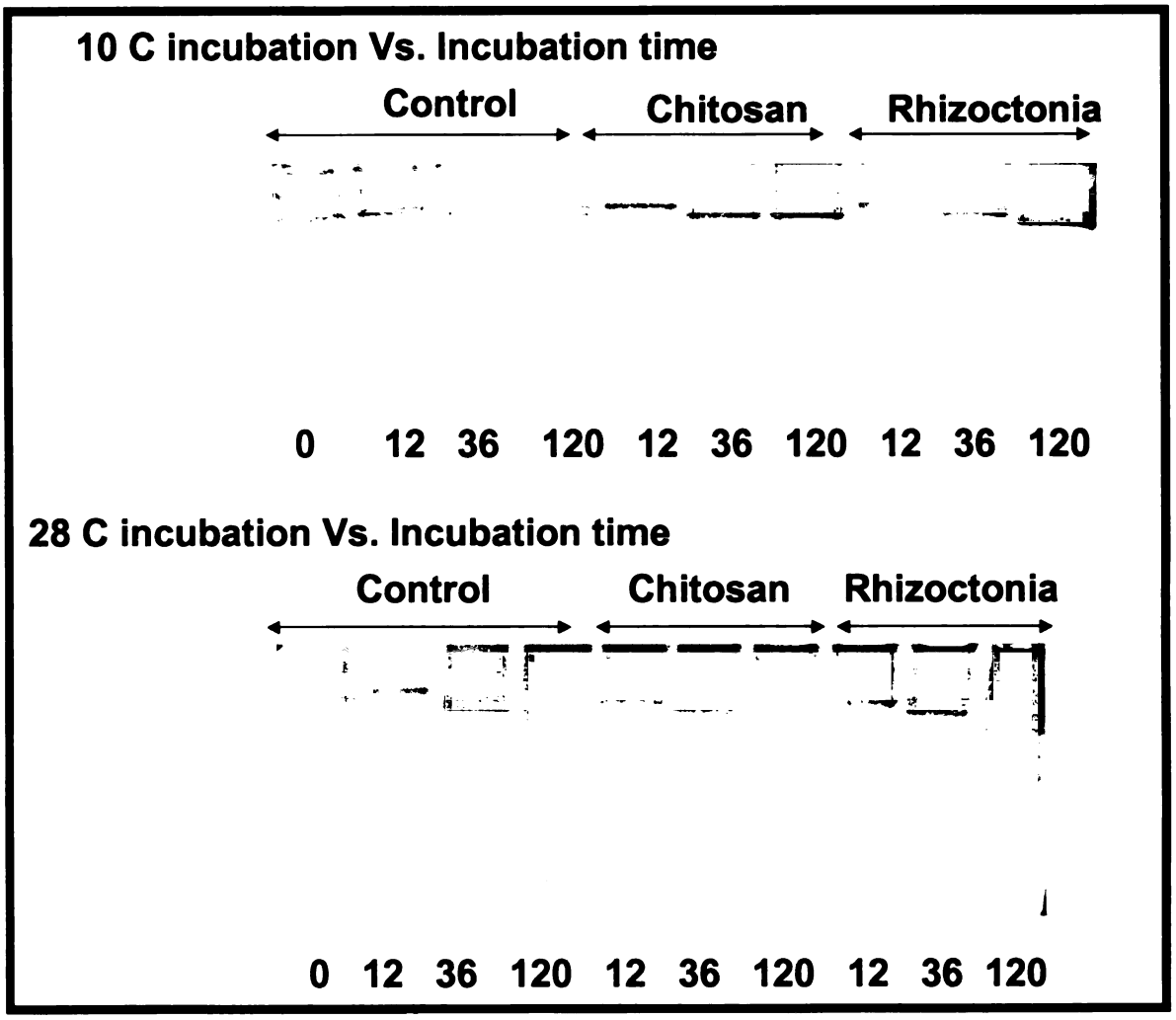

Figure 2.10: Incubation temperature Vs. Incubation time .Native gel electrophoresis of proteins prepared from sugarbeet tap roots collected after inoculation with water (control) Chitosan and *Rhizoctonia solani* AG2-2 and incubated at 10°C (top) and 28°C (bottom) for 0,12, 36 and 120 hours. The gel was stained for chitinase hydrolysis.

DISCUSSION

Based on the intensity of the bands, overall activity of chitinases in sugar beet tap root tissues was greater at 28°C than at 10°C. This is in contrast to the previously observed defense response involving production of phenolic compounds that was expressed only at the cooler temperature (Halloin 1994). Enhanced production of chitinase enzyme, even in water treated control tissues suggests that this production is part of a non-specific wound response to treatments.

The present studies with the control (water), *R.solani* and chitosan treatments have demonstrated that chitinases are induced in sugar beet tap roots (susceptible cultivar E17) in response to infection by fungal pathogen, an abiotic inducer- chitosan and wounding at two different incubation temperatures10˚ C and 28˚ C. At zero time (0 hours) incubation one high intensece chitinase band and a very low intense chitinase isozyme band are found. When the incubation time increased to 36 hours or more, three or more isozymes were induced. Compared to control, the tissues treated with *Rhizoctonia* and chitosan and incubated at 28˚ C and the tissues treated with chitosan and incubated at 10˚ C had consistently high intense bands up to 72 hours of incubation. This high intense banding pattern was not seen for tissues treated with *Rhizoctonia* and incubated at 10˚ C during first 72 hours of incubation. It is not known whether

R.solani ramify the host tissue at a low rate at 10˚ C incubation compare to that of 28˚ C incubation or sugar beet-*R.solani*- the host-pathogen interaction at 10˚ C do not cause the chitinase induction. When the incubation time is increased to 96 hours, 120 hours or 144 hours the bands are smeared. This may be due to tissue maceration during prolonged incubation. It is unknown if similar chitinase induction occurs in resistant cultivars when infected with *Rhizoctonia solani*.

Chitinases are hypothesized to protect the plant from the fungal pathogens in two different ways: the chitinase enzymes are highly potent growth inhibitors of certain fungal pathogens (Schlumbanum *et. al.* 1986) and do this by attacking the fungal cell wall component chitin. The second mode of action is that their action can release oligo-saccharide elicitors from fungal cell walls that can activate a variety of plant defenses (Lawrence *et. al* 2000). Even though our experimental results show that induction of chitinases is likely a generalized wound response, this still could be one of the first lines of defense that the plants express. In the presence of fungal pathogens, induced chitinase may attack the cell wall of those fungi and release the fungal cell wall components as elicitors, which in turn may activate other plant defense responses. Temperature may play a vital role in an elicitor-induced defense response pathway.

Boller *et al.* (1983) reported that in a system with a closed atmosphere, chitinase was induced in bean leaves by wound ethylene, which accumulated in the atmosphere. Continuous presence of ethylene was necessary for full induction of chitinases. In our experimental system we used perforated bags to

store the tissue blocks for incubation to avoid ethylene accumulation, but wounding to form inoculation hole and incubating in closed growth chamber may have provided necessary conditions for chitinase induction by wound ethylene. The high chitinase activity observed in control (water) treatments may be caused by wound ethylene.

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CHAPTER THREE

Current status and future direction of chitinases in sugar beets

Current status and future direction of chitinases in sugar beets

Pathogenesis-related (PR) proteins are thought to play an important role in plant defense against invading organisms. A number of different PR-proteins have been identified (Hammond and Jones 2000). There are several reports showing positive correlation between the expression of PR-genes and disease resistance. In an effort to enhance the disease resistance, PR proteins have been induced in plants either using abiotic inducers or constitutively expressing the protein. Some plant chitinases and β-1,3-glucanases are PR proteins. These enzymes can hydrolyze the fungal cell wall. These enzymes were shown to be induced in plants upon infection and some purified proteins were observed to have antifungal activity in vitro. Chitinases have been purified and characterized from a number of plant sources. Genes encoding these enzymes have also been cloned from a variety of plant sources. Currently, there is an immense interest in delineating the molecular events from pathogen recognition to the expression of these genes.

Different classes of endo-chitinases are localized and accumulated in different parts the plant tissue (Verburg and Huynh 1995). For example vacuolar localization or intercellular localization. Even though our results suggest that chitinase induction during Rhizoctonia root and crown rot disease is a generalized wound response, it would be important to ascertain if any particular class of endo-chitinase is differentially induced during sugar beet-*R.solani* interaction under warm (>20°C) or cool (20 °C>) temperature or there is a

particular spatial distribution of endo-chitinase during Rhizoctonia root and crown rot disease at the different temperatures. Chitinase isozyme bands are showing different migration levels on the polyacrylamide gels under native conditions. For future experiments it would be interesting to know why these protein bands are migrating differently. The specific size, shape or charge difference among the different chitinase isozymes that were induced during sugar beet-Rhizoctonia or sugar beet-chitosan (abiotic inducer) interaction may be critical in its function as PR proteins.There are sugar beet breeding lines that are resistant to Rhizoctonia crown and root rot. We should ascertain how chitinases are induced in sugar beet breeding lines that are resistant to Rhizoctonia root and crown rot disease.

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