



DISSERTATION

**EFFECTS OF CHITOSAN ON AGRONOMIC CHARACTERS AND SOME
PHYSIOLOGICAL RESPONSES OF RICE (*Oryza sativa* L.)
CULTIVAR SUPHANBURI 1 UNDER DROUGHT CONDITION**

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GRADUATE SCHOOL, KASETSART UNIVERSITY

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THESIS

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Chitosan is a natural biopolymer extracted from chitin by deacetylation process. Chitosan not only stimulates growth and increases yield but also regulates the immune system of plants under unfavorable conditions. The objectives of this study were to determine the effective type of chitosan and the appropriate application for rice yield increasing under drought condition. A series of experiments were carried out in order to obtain basic information about chitosan application in rice plants. This study was conducted in a greenhouse and laboratory at Rajamangala University of Technology Suvarnabhumi, Huntra Campus, during November 2003 to February 2006. The results found that the application of polymeric chitosan by seed soaking before planting followed by four foliar sprayings showed the best effect on growth and rice yield. The optimum chitosan concentration for applying in rice plants was 20 ppm, and four foliar sprayings throughout cropping season was the best effectiveness. Under drought condition, it indicated that rice plants applied with chitosan before drought occurrence showed good recovery and maintained growth and yield over those of control. Some physiological responses of rice plants under drought after chitosan application showed that the proline and soluble sugar accumulation in treatment applied with chitosan and fertilizer rapidly increased over the control whereas the activity of nitrate reductase tended to be higher than the others but did not show any significant differences. The total nitrogen content in treatment applied with chitosan and fertilizer was significantly increased over the control. In this study, it may be concluded that application of polymeric chitosan at the rate of 20 ppm sprayed before drought might reduce the severe damage of rice plants and maintaining growth and yield.

Student's signature

Thesis Advisor's signature

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LIST OF ABBREVIATIONS AND ACRONYMS

DA	Deacetylation
DD	Degree of deacetylation
KDa	Kilo daltons
LS D	Least significant difference
SES	Standard evaluation system
IRRI	International Rice Research Institute
DAS	Day after sowing
ABA	Abscisic acid
PAL	Phenylalanine ammonia lyase
TAL	Tyrosine ammonia lyase
RWC	Relative water content
NR	Nitrate reductase
SI	Sufficient irrigation
D	Drought without chitosan
CbD	Chitosan sprayed before drought
CdD	Chitosan sprayed during drought
CaD	Chitosan sprayed after drought
Aval. P	Available phosphorous
Exch.K	Exchangeable potassium
C.V.	Cultivar
SS	Seed soaking
SS+FPR	Seed soaking and foliar spray
FPR	Foliar spray
w/v	Weight by volume
WUE	Water use efficiency
WBC	Water binding capacity
FBC	Fat binding capacity

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INTRODUCTION

Chitosan is a natural biopolymer extracted from chitin, which is the main structural component of shells of shrimps and crabs, squid pens and cell walls of some fungi. Chitin and chitosan are copolymers found together in nature. They especially have an inherent property of being environmentally friendly and easily degradable. Thailand is a world-leading exporter of frozen shrimps, therefore, there are abundant raw material for chitosan production, which is a way to create added value to the material. Chitosan has a wide scope of application on various plants. With high affinity and non-toxicity, it does no harm to human beings and livestock. Chitosan regulates the immune system of plants, and induces the excretion of resistant enzymes. Therefore, chitosan not only activates the cells and stimulates the growth of plants, but also improves its disease and insect resistant ability (Doares *et al.*, 1995). Importantly, chitosan inhibits the reproduction of pathogens. Besides, chitosan is an elicitor of plant defense mechanism by reducing stomatal opening and stimulating stomatal closing under unfavorable conditions (Ganesh *et al.*, 2002).

Chitosan has a great effect on agriculture including being the carbon source for microbes in the soil, accelerating the transferring process of organic matter into inorganic matter and facilitating the root system of plants to absorb more nutrition from the soil. Chitosan is absorbed to the root after being decomposed by bacteria in the soil and chitin enzymes secreted by the roots (Somashekar and Richard, 1996; Brian *et al.*, 2004). The plants with high content of chitin enzyme have better external disease resistance to pathogen than others (Khan *et al.*, 2003). Using chitosan in agriculture, even without applying chemical fertilizer, can increase the microbial population in a large numbers, and transforms organic nutrient into inorganic nutrient, which can be easily absorbed by the plant roots, so that the nutrient can be fully absorbed by the plant. A high chitin enzyme level improves the durability and resistance of the plant, resulting in it being difficult to be infected by germs, then not proliferating even if infected, and curing the disease by itself (Synowiecki and Nadia, 2003). Chitosan application with less chemical fertilizer uses increases the production by 15-20% according to the kinds of plant (Hong *et al.*, 1998).

Rice (*Oryza sativa* L.) is the main staple food in Thailand. The production of rice has not only created employment opportunities for one billion agricultural workers in developing nations, but has also contributed to developing the splendid culture associated with rice production. Hence, effective and productive rice systems play an influential role in the development of economic and improvement of life quality. Thailand is one of rice producers, and also the biggest rice exporter. The world rice consumption has increased for the last three years. In conjunction with the world rising consumption level, the rice production will expand in order to meet this

higher demand. But there are many problems in rice production. One of the important problems is drought which dramatically limits rice productivity because some processes associated with plant growth are affected. Therefore, this problem need to be immediately solved due to its seriousness relating to human beings. A good way to reduce the severity of damage to rice plants from drought is to induce them to produce some metabolites which are associated with drought tolerance. Chitosan is a biopolymer which has been reported to trigger some enzymes such as phenylalanine ammonia-lyase (PAL) and tylosine ammonia-lyase (TAL) associated with producing secondary metabolites resulting in self -defense (Loscke *et al.*,1983). Lee *et al.*(1999) reported that chitosan reduced the size of stomatal aperture and inhibited light-induced stomatal opening by inducing reactive oxygen species (ROS) including super oxide and hydrogen peroxide which inhibit stomatal opening and promote stomatal closing.

OBJECTIVES

1. To determine the most effective type of chitosan and appropriate application method for increasing rice yield.
2. To determine the optimum concentration and timing of chitosan application for increasing rice yield.
3. To study drought recovery and rice yield potential after chitosan application under drought condition.
4. To study some physiological responses of rice after chitosan application under drought condition.

LITERATURE REVIEW

1. History of chitin and chitosan

The first works on chitin mentioned in the literature are those of an English researcher, Hachett. In 1799, he reported that after demineralisation by acidic treatment, crustacean shells maintained their physical properties. The discovery of chitin is due to a French botanist, Henri Braconnot. In 1911, he published very interesting results obtained from the experiments he did on six different varieties of higher fungi. He isolated a new and quite distinct substance, which was different from the woody material of plants. This substance was certainly not pure chitin but he gave very precise information on the chemical structure of this new molecule he termed "Fungine". He mentioned in particular the presence of nitrogen and the liberation of potassium acetate in the course of its thermal decomposition in the presence of concentrated potassium hydroxide and thus was not so far from the discovery of chitosan. Chitin was then discovered approximately 30 years before cellulose. In 1823, another French scientist, A. Odier isolated chitin from the elitrium of the May-bug. Surprisingly, he failed to find nitrogen in its structure and ignored completely the works of Braconnot. Nevertheless, he had the merit to find its actual name, chitine, from the Greek $\chi\iota\tau\omega\sigma$ which means tunic or coverage. In 1824, J.G. Children, an English researcher translated in English the paper of A.Odier and confirmed the presence of nitrogen in the chemical structure. The discovery of chitosan was obtained in 1859 by another French scientist, C. Rouget. He reported that after the treatment of chitin in hot and concentrated potassium hydroxide, he obtained a new substance soluble in dilute acidic aqueous solutions. He named it "modified chitin". In 1894, F. Hoppe Seyler attributed the actual name, chitosan, to this new substance and, surprisingly ignored the existence of Rouget. Unfortunately, this name is at the origin of a very difficult problem of nomenclature. Chitin and chitosan are both copolymers of the same repeating units and, as a consequence only one name, should be used for these same polymers. In 1928, K.H. Meyer and H. Mark proposed for the first time, the structure of pyranose ring linked β , (1 \rightarrow 4) as the repeating unit of chitin chains. His description of the chemical structure of chitin was completely achieved in 1939 by A. Neuberger and R. Pitt Rivers. They gave the exact structure of the pyranose ring of N-acetylglucosamine with the amino group on the c₂. The crystallographic structure, in particular the presence of three different polymorphs corresponding to chitins α , β and γ was described for the first time by K.M. Rudall in 1963 (Domard,1996).

2. Definition and chemical properties

Chitin is one of the most abundant organic materials, beings second only to cellulose in the amount produced annually by biosynthesis. It occurs in animals, particularly in crustacea, mollouses and insects, where it is a major constituent of the exoskeleton, and in certain fungi, where it is the principal fibrillar polymer in the cell

wall. Chitin has a crystalline structure and it constitutes a network of organized fibers. This structure confers rigidity and resistance to organisms that contain it (Roberts,1992).Chitin is poly [β (1 \rightarrow 4)-2-acetamido-2-deoxy-D-glucopyranose] and its idealised structure is shown in Figure 1. Crystalline chitin is insoluble in ordinary solvent, water, dilute acid and concentrated alkalis (Austin *et al.*,1981, Muzzarelli,1977). It is soluble in conc. HCl, H₂SO₄ 70-97%, H₃PO₄ and anhydrous formic acid (Deshpande,1986). Chitin can be reprecipitated from these salts by dilution with alcohol or acetate yielding continuous filaments (Austin *et al.*,1981).

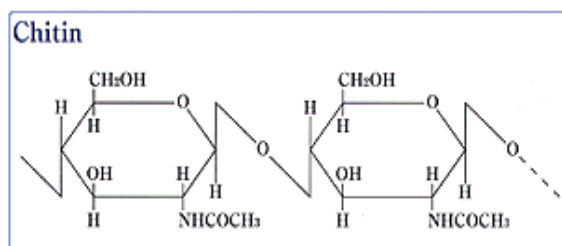


Figure 1. Chemical structure of chitin (Roberts, 1992).

The principal derivative of chitin is chitosan, produced by alkaline deacetylation of chitin. Chitosan also occurs naturally in some fungi but its occurrence is much less widespread than that of chitin. Chitosan is poly [β (1 \rightarrow 4)-2-amino-2-deoxy-D-glucopyranose] and its idealized structure is shown in Figure 2. Chitosan is insoluble in water, concentrated acid, alkali, alcohol and acetone. It is insoluble in any common organic solvent (e.g. dimethylformamide and dimethylsulfoxide). Chitosan is soluble in dilute nitric acid, acetic acid and formic acid. The best solvent for chitosan is formic acid solution containing 0.2 to 100% of formic acid (Knorr,1984).

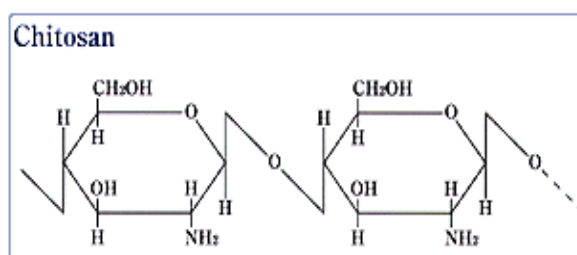


Figure 2.Chemical structure of chitosan (Roberts,1992).

The typical commercial chitosan has approximately 85 % degree of deacetylation (85%DD).Thus the terms chitin and chitosan describe a continuum of copolymers of N-acetyl-D-glucosamine and D-glucosamine residues, the two being distinguished by insolubility or solubility in diluted aqueous acid solution. Their properties are frequently dependent on the relative proportions of N-acetyl-D-glucosamine and D-glucosamine residues (Roberts, 1992).

3. Chitosan preparation

Chitosan is extracted from crustacean shell waste such as crab, shrimp, lobster and crawfish. The shells contain approximately 30-40% protein, 30-50% calcium carbonate and 20-30% chitin on a dry basis (Johnson and Peniston, 1982). These portions vary with crustacean species and seasons (Green and Mattick, 1979). Isolation of chitosan from crustacean shell waste involves four steps: deproteinization (DP), demineralization (DM), decolorization (DC) and deacetylation (DA)

3.1 Deproteinization

Chitin occurs naturally in association with protein (chitinoprotein). Some of this protein can be extracted by mild methods, but other parts are not readily extracted, suggesting strong covalent bonding to chitin (Attwood and Zola, 1967). With regards to chemical structure, protein is bound by covalent bonds to the chitin through aspartyl or histidyl residues, or both, thus forming stable complexes such as glycoprotein.

Crustacean shell waste is usually ground and treated with dilute sodium hydroxide solution (1-10%) at elevated temperature (65-100°C) to dissolve the proteins present. Reaction time usually ranges from 0.5 to 12 hr depending on preparation method. Prolonged alkaline treatment under severe conditions causes depolymerization and deacetylation. To obtain uniformity in reaction, it is recommended to use relatively high ratios of solid to alkali solution of 1:10 or 1:15-20 with proper agitation because a minimum ratio of 1:4 (w/v) of shell weight to KOH solution has only a minor effect on the DP efficiency of shells (No and Meyers, 1995). Optimal conditions for deproteinization involve treatment of the crawfish shells with 3.5% (w/w) NaOH solution for 2 hr at 65°C with constant stirring and a solid to solvent ratio of 1:10 (w/v) (No *et al.*, 1989).

During the deproteinization process, foam formation can occur, but the foam is not as brisk and intense as that produced during demineralization. Shahidi and Synowiecki (1991) suggested that the optimal deproteinization can be achieved by using dilute potassium hydroxide solution. Generally, if maximizing protein yield and quality is the objective, protein extraction before demineralization is recommended (Johnson and Peniston, 1982) or the pH of the aqueous solution must be reduced to the isoelectric point of protein for precipitation (Green and Mattick, 1979).

3.2 Demineralization

Demineralization is usually accomplished by extraction with dilute hydrochloric acid (up to 10%) at room temperature with agitation to dissolve calcium carbonate. The use of HCl acid at higher concentration and also 90% formic acid to achieve demineralization has been reported. Optimum demineralization is achieved by constant stirring of the dried ground shell with 1 N HCl for 30 min at ambient temperature and a solid to solvent ratio of 1:15 (w/v) (No *et al.*, 1989). The ash content of the demineralized shell is an indicator of the effectiveness of the demineralization process. Elimination of the demineralization results in products having 31-36% ash.

During the demineralization process excessive undesirable foams are produced due to CO₂ generation ($[\text{CaCO}_3 + 2\text{HCl} \rightarrow \text{CaCl}_2 (\uparrow) + \text{H}_2\text{O}]$). To control or reduce the foam, No and Hur (1998) recommended the use of commercial antifoam comprising of 10% solution of active silicone polymer without an emulsifier. They also demonstrated that at 1.0 ml of antifoam /L of 1N HCl, the performance of antifoam is more efficient during demineralization with smaller shell particle size (<0.425 mm) and under a slightly faster stirring speed (300 rpm). Furthermore, they recommended that deproteinization followed by demineralization is a favorable sequence in terms of the amount of antifoam required to control foaming.

3.3 Decoloration

Acid and alkali treatment alone produce a colored chitin product. For commercial acceptability, the chitin produced from crustacean sources, needs to be decolorized or bleached to yield cream white chitin powder (No *et al.*, 1989). The pigment in the crustacean shells forms complexes with chitin. In an earlier study, Fox (1973) found 4-keto-and three 4,4-diketo- β -carotene derivatives firmly bound to exoskeleton chitin of kelp crab. The level of association of chitin and pigments varies from species to species among crustacean.

Several workers have used reagents to eliminate pigments from crustacean exoskeleton, usually crab. However, with crawfish shell the reagents alone do not seem with effective as the procedure developed currently. This suggests that carotenoids are more strongly bound to the crawfish shell matrix than are those reported from other crustacean (No *et al.*, 1989). Hence, the stronger the bond, the more harsh treatment is required to prepare a white colored chitin. During the process of decoloration, it should be noted that the chemical used should not affect the physicochemical or functional properties of chitin and chitosan. No *et al.* (1989) was able to prepare a near white colored crawfish chitin by extraction with acetone and drying for 2 hr at ambient temperature, followed by bleaching with 0.315% (v/v) sodium hypochloride solution (containing 5.25% available chlorine) for 5 min with a solid to solvent ratio of 1:10 (w/v) based on dry shell. However, the color of chitin products varied from cream white to intermediate pink color. Without prior acetone

extraction, bleaching for more than 1 hr was needed to obtain a commercially acceptable white product.

3.4 Deacetylation

Deacetylation is the process to convert chitin to chitosan by removal of acetyl group. It is generally achieved by treatment with concentrated sodium or potassium hydroxide solution (40-50%), usually at 100°C or higher, for 30 min or longer to remove some or all of the acetyl groups from the polymer (No and Meyers, 1985). The N-acetyl groups cannot be removed by acidic reagents without hydrolysis of the polysaccharide, thus, alkaline methods must be employed for N-deacetylation (Muzzarelli, 1977).

Depending upon the production sequence, deacetylation can be achieved by reaction of demineralized shells or crawfish with 50% NaOH (w/w) solution at 100°C in air using a solid to solvent ratio of 1:1 (w/v) (No *et al.*, 1989). There are several critical factors that affect the extent of deacetylation including temperature and time of deacetylation, alkali concentration, prior treatment applied to chitin isolation, atmosphere (air or nitrogen), ratio of chitin to alkali solution, density of the chitin, and particle size (Rigby, 1936). Considering all these as necessary conditions, the ideal purpose of deacetylation is to prepare chitosan that is not degraded and is soluble in dilute acid in minimal time.

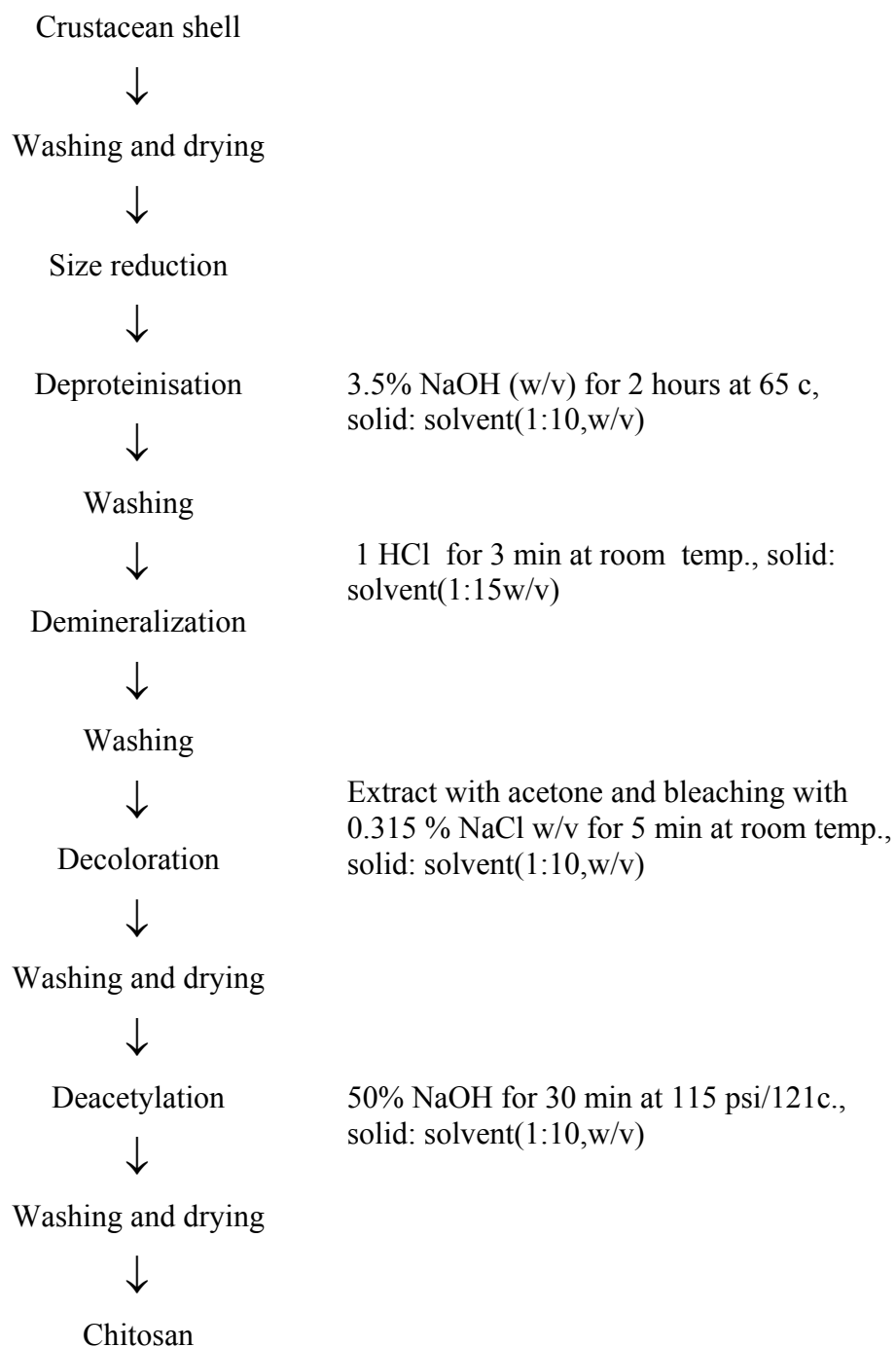


Figure 3 Simplified flow diagram of chitin and chitosan processing (Knorr ,1984).

4. Factors affecting chitosan production

A number of processing factors affect chitosan's physicochemical characteristics.

4.1 Temperature of deacetylation

Higher temperature tends to increase the degree of deacetylation but reduces molecular size (Lusena and Rose, 1953). There is a substantially linear relationship between temperature and the rate of deacetylation (Peniston and Johnson, 1980).

4.2 Time of deacetylation and alkali concentration

Wu and Bough (1978) suggested that deacetylation proceeds rapidly to about 68% during the first hour in 50% NaOH solution at 100°C. However, the reaction progresses gradually thereafter reaching about 78% in 5 hour. Thus, alkali treatment beyond 2 hour does not deacetylate chitin significantly, rather it degrades the molecular chain. In a concentration study with 35, 40, and 50% NaOH, Bough *et al.* (1978) found that as alkali concentration decreased, rates of decrease in both viscosity and molecular weight distribution also slowed. They concluded that chitosan deacetylated for 5 min with 50% NaOH at 145-150°C had higher viscosities (1.7-16.4 flod) and molecular weight (1.1-1.8 fold) than did chitosans deacetylated for 15 min.

4.3 Effect of treatment conditions applied to chitin isolation

Treatment conditions applied to chitosan isolation primarily affect viscosity of the product more than any other property. The use of HCl at concentrations above 1.25N adversely affects the viscosity of the final chitosan product. In addition, chitosan viscosity tends to decrease with increased time of demineralization (Moorjani *et al.*, 1975). On the other hand, Bough *et al.* (1978) found that deproteinization with 3% NaOH, and elimination of the demineralization step in chitin preparation, decreased the viscosities of chitosan samples, whereas Moorjani *et al.* (1975) indicated that it is not desirable to bleach the material at any stage since bleaching considerably reduces the viscosity of the final chitosan product.

4.4 Atmosphere

Many scientists have agreed that free access of oxygen to chitin during deacetylation has a substantial degrading effect on chitosan. Deacetylation in the presence of nitrogen yielded chitosan of higher viscosity and molecular weight distributions than in air. However, little differences in nitrogen and ash compositions were observed (Bough *et al.*, 1978; Lusena and Rose, 1953; Rigby, 1936).

4.5 Ratio of chitin to alkali solution

Moorjani *et al.* (1975) emphasized that ratio of chitin solids to alkali solution plays a significant role in determining the quality of chitosan, based on viscosity determination. The reported solid to solution ratios range from 1:10 to 1:100 on a wet basis, and 1:4 on a dry basis or when dry heating is used.

4.6 Particle size

Particle size in chitosan production has sparked controversial reports on its effect on chitosan quality. Some agree that small particle size is better than large particle size. According to Bough *et al.* (1978), smaller particle size (1 mm) results in a chitosan product of both higher viscosity and molecular weight than that of larger particle size (above 2 to 6.4 mm). The larger particle sizes require longer swelling time resulting in a slower deacetylation rate. However, Lusena and Rose (1953) indicated that size of chitin particle within the 20-80 mesh range (0.841-0.177 mm) had no effect on the extent of deacetylation and viscosity of the chitosan solutions.

5. Characteristics of chitosan

5.1 Degree of deacetylation (DD)

The process of deacetylation involves the removal of acetyl groups from the molecular chain of chitin, leaving behind a compound (chitosan) with a high degree of chemical reactive amino group (-NH₂). This makes the degree of deacetylation (DD) an important property in chitosan production as it affects the physicochemical properties, and hence determines in appropriate applications (Rout, 2001). Deacetylation also affects the biodegradability and immunological activity (Tolaimate *et al.*, 2000). The degree of deacetylation can be employed to differentiate between chitin and chitosan because it determines the content of free amino groups in the polysaccharides. In fact, there are two advantages of chitosan over chitin. In order to dissolve chitin, highly toxic solvents such as lithium chloride and dimethylacetamide are used whereas chitosan readily dissolves in diluted acetic acid. The second advantage is that chitosan possesses free amine groups which are an active site in many chemical reactions. The degree of deacetylation of chitosan ranges from 56% to 99% with an average of 80% depending on the crustacean and the preparation methods (No *et al.*, 2000 ; No and Meyers, 1995). Chitin with a degree of deacetylation of 75% or above is generally known as chitosan. Various methods have been reported for the determination of the degree of deacetylation of chitosan. These include ninhydrin test, linear potentiometric titration, near-infrared spectroscopy, and first derivative UV-spectrophotometry (Khan *et al.*, 2002).

The IR spectroscopy method, which was first proposed by Moore and Roberts (1980), is commonly used for the estimation of chitosan DD values. This method has a number of advantages and disadvantages. First, it is relatively fast and unlike other

spectroscopic methods, does not require purity of the sample to be tested nor require dissolution of the chitosan sample in an aqueous solvent. However, the IR method utilizing baseline for DD calculation may be a possible argument for employment of different baseline which would inevitably contribute to variation in the DD values. Secondly, sample preparation, type of instrument used and conditions may influence the sample analysis. Since chitosan is hygroscopic in nature and samples with lower DD may absorb more moisture than those with higher DD, it is essential that sample analysis must be completely dry (Khan *et al.*, 2001; Blair *et al.*, 1987).

5.2 Viscosity

Viscosity is an important factor in the conventional determination of molecular weight of chitosan and in determining its commercial applications in complex biological environments such as in the food system. Higher molecular weight chitosan often render highly viscous solutions, which may not be desirable for industrial handling. Some factors during processing such as the degree of deacetylation, molecular weight, concentration of solution, ionic strength, pH, and temperature affect the production of chitosan and its properties. For instance, chitosan viscosity decreases with an increased time of demineralization (Moorjani *et al.*, 1975). Viscosity of chitosan in acetic acid tends to increase with decreasing pH but decrease with decreasing pH in HCl, giving rise to the definition of 'Intrinsic Viscosity of chitosan, which is a function of the degree of ionization as well as ion strength. Bough *et al.* (1978) found that deproteinization with 3% NaOH and elimination of the demineralization step in the chitin preparation decreased the viscosity of the final chitosan products. Moorjani *et al.* (1975) also stated that it is not desirable to bleach the material (i.e., bleaching with acetone or sodium hypochlorite) at any stage since bleaching considerably reduces the viscosity of the final chitosan product. Similarly, No *et al.* (2000) demonstrated that chitosan viscosity is considerably affected by physical (grinding, heating, autoclaving, ultrasonication) and chemical (ozone) treatment, except for freezing, and decreases with an increase in treatment time and temperature. Chitosan solution stored at 4 ° C is found to be relatively stable from a viscosity point of view.

The effect of particle size on the quality of chitosan products was investigated by Bough *et al.* (1978), who reported that smaller particle size (1mm) results in chitosan products of both higher viscosity and molecular weight than those of either 2 or 6.4 mm particle size. They further enumerated that a larger particle size requires longer swelling time, resulting in a slower deacetylation rate. But, in contrast, Lusena and Rose (1953) reported that the size of chitin particle within the 20-80 mesh (0.841-0.117 mm) range had no effect on the viscosity of the chitosan solutions.

5.3 Solubility

While chitin is insoluble in most organic solvents, chitosan is readily soluble in dilute acidic solutions below pH 6.0. Organic acids such as acetic, formic, and lactic acids are used for dissolving chitosan. The most commonly used is 1% acetic acid solution at about pH 4.0 as a reference. Chitosan is also soluble in 1% hydrochloric acid but insoluble in sulfuric and phosphoric acids. Solubility of chitosan in inorganic acids is quite limited. Concentrated acetic acid solutions at high temperature can cause depolymerization of chitosan (Roberts and Domszy, 1982). Above pH 7.0 chitosan's solubility stability is poor. At higher pH, precipitation or gelation tends to occur and the chitosan solution forms poly-ion complexes with anionic hydrocolloid resulting in the gel formation (Kurita, 1998). The concentration ratio between chitosan and acid is very important to impart desired functionality (Mima, 1983). At concentrations as high as 50 percent organic solvent, chitosan still works as a viscosifier causing the solution to remain smooth. There are several critical factors affecting chitosan solubility including temperature and time of deacetylation, alkali concentration, prior treatments applied to chitin isolation, ratio of chitin to alkali solution and particle size.

The solubility, however, is controlled by the degree of deacetylation and it is estimated that deacetylation must be at least 85 % complete in order to achieve the desired solubility (No and Meyer, 1995). The acid-soluble chitosan with >95% solubility in 1% acetic acid at a 0.5% concentration could be obtained by treatment of the original chitin with 45-50% NaOH for 10-30 min. Chitosan treated with 45% NaOH for only 5 min, and/or with 40% NaOH for 30 min, were not deacetylated sufficiently to be soluble in 1% acetic acid. Insoluble particles were found in both solutions. According to Bough *et al.* (1978), a reaction time of 5 min with 45% NaOH may not be enough for chitin particles to be sufficiently swollen. A decrease of the NaOH concentration to 40% required increased time of >30 min to obtain a soluble chitosan (No *et al.*, 2000).

5.4 Bulk Density

The bulk density of chitin from shrimp and crab is normally between 0.06 and 0.17 g/ml, respectively (Shahidi and Synowiecki, 1991), indicating that shrimp chitin is more porous than crab chitin. In a study conducted by Rout (2001), the bulk density of chitin and chitosan from crawfish shell, was very high (0.39 g/cm³). This was calculated as an unpacked bulk density of chitosan particles passed through a 0.5 mm mesh into a 25 ml measuring cylinder. This perhaps could be due to the porosity of the material before treatment. A comparison of the bulk densities of crawfish and commercial chitin and chitosan indicated same variations that can be attributed to crustacean species or sources of chitosan and the methods of preparation (Rout, 2001) as also stated earlier by Brine and Austin (1981). Rout (2001) reported that increased degree of deacetylation (DD) decreased bulk density.

5.5 Color

The pigment in the crustacean shells forms chitin complexes (4-keto and three 4, 4'-diketo -B - carotene derivatives) (Rout, 2001). Chitosan powder is quite flabby in nature and its color varies from pale yellow to white whereas starch and cellulose powder have smooth texture and white color.

5.6 Water binding capacity (WBC) and Fat binding capacity (FBC)

Water uptake of chitosan is significantly greater than that of cellulose and even chitin (Knorr, 1982). Basically, WBC for chitosan ranges between 581 to 1150% with an average of 702%. It has been also noted that reversing the sequence of steps such as demineralization (DM) and deproteinization (DP) had a pronounced effect on WBC and FBC. DP of demineralized shell also gives higher WBC compared to the process when DM of the deproteinized shell is conducted. Besides, the process of decoloration (DC) also causes a greater decrease in WBC of chitosan than those of unbleached crawfish chitosan (Rout,2001).

The fat uptake of chitin and chitosan ranges from 170 to 315 % with chitosan having the lowest and chitin the highest fat uptake (Knorr,1982). In a study by Rout (2001) on this aspect, the average FBC of crawfish chitosan and commercial crab chitosan for soybean oil were 706% and 587%, respectively. The inclusion of decoloration step during the production of chitosan was found to decrease the fat binding capacity of crawfish chitosan, and decoloration (bleaching) had been shown to affect the viscosity of chitosan (Moorjani *et al.*, 1975). The decreased viscosity as evidenced may be a cause for decrease in fat binding capacities among unbleached and bleached crawfish chitosan samples. Rout (2001) also reported that changing the sequence of steps, i.e., when demineralization (DM) was conducted prior to deproteinization (DP), followed by deacetylation (DA), caused an increase in FBC compared with when deproteinization (DP) was performed prior to demineralization (DM), followed by deacetylation (DA).

5.7 Emulsification

Chitosan alone does not produce emulsions. Cho *et al.* (1998) reported that the emulsifying capacity of egg yolk increased with the addition of chitosan compared with the control. At 0.5% chitosan concentration, better emulsions tend to be very stable under temperature changes and aging. With viscosity, the degree of deacetylation is reported to be a determining factor in the emulsification properties of chitosan. Protein solution containing chitosan with intermediate DD produces less effective emulsion compared with that containing chitosan with higher DD (Rout , 2001).

5.8 Antimicrobial properties

Recent studies in antibacterial activity of chitosan have revealed that chitosan is effective in inhibiting bacterial growth. The antimicrobial properties of chitosan depend on its molecular weight and the bacterial types. For gram-positive bacteria, chitosan with 470 KDa was the most effective, except for *Lactobacillus sp.*, whereas for gram-negative bacteria, chitosan with 1,106 KDa was effective. Chitosan generally showed stronger bactericidal effects for gram-positive bacteria (*Listeria monocytogenes*, *Bacillus megaterium*, *B. cereus*, *Staphylococcus aureus*, *Lactobacillus plantarum*, *L. brevis*, and *L. bulgaris*) than for gram-negative bacteria (*E.coli*, *Pseudomonas fluorescens*, *Salmonella typhimurium*, and *Vibrio parahaemolyticus*) in the presence of 0.1% chitosan (No *et al.*, 2000). Koide (1998) reported that chitin and chitosan in vitro showed antibacterial and anti-yeast activities. One of the chitosan derivatives, N-carboxybutyl chitosan, was tested against 298 cultures of different pathogenic microorganisms that showed bacteriostatic and bactericidal activities, and there were marked morphological alterations in treated microorganisms when examined by electron microscopy (Muzzarelli *et al.*, 1990). Conversely, growth inhibition and inactivation of mould and yeasts seem to depend on chitosan concentration, pH and temperature (Rout, 2001). According to Cuero (1999), the antimicrobial action of chitosan is influenced by intrinsic and extrinsic factors such as the type of chitosan (e.g., plain or derivative), degree of chitosan polymerization, host nutrient constituency, substrate chemical and/or nutrient composition, and environmental conditions. Tsai *et al.* (1999) studied the antimicrobial activity of chitosan prepared from shrimp against *E.coli*, and found that higher temperature and acidic pH of foods increased the bactericidal effect of chitosan. They also explained the mechanism of chitosan antibacterial action involving a cross-linkage between polycations of chitosan and the anions on the bacterial surface that changes membrane permeability.

Chitosan coating has been shown to significantly delay fruit spoilage or decaying of fruits and vegetables such as tomatoes, strawberries, etc., at different temperatures. Chitosan coated fruits were not only firmer and higher in titratable acidity, but were slow to decay and exhibited less pigmentation than control samples at the end of storage (El *et al.*, 1992). Low molecular weight chitosan has a greater inhibitory effect against phytopathogens than the high molecular weight chitosan (Hirano *et al.*, 1989).

5.9 Molecular weight

Knowledge of molecular weight of chitosan is very important for industrial uses and for advanced research in numerous fields. It varies with the raw material sources and method of preparation. Molecular weight of native chitin is usually larger than one million Daltons while commercial chitosan products have the molecular weight range of 100,000-1,200,000 Daltons, depending on the process and grade of the product (Li *et al.*, 1992). In general, high temperature, dissolved oxygen, and shear stress can cause degradation of chitosan. For instance at a temperature over 280

c^o, thermal degradation of chitosan occurs and polymer chains rapidly break down, thereby lowering molecular weight (Rout, 2001). Also, maximal depolymerization caused by utilization of high temperature or concentrated acids, such as hydrochloric acid followed by acetic acid and sulfurous acid, results in molecular weight changes with minimal degradation with the use of EDTA (Rout, 2001).

6. Drought stress affecting plant responses

Hall (1940) defined that drought is where a dry soil (due to lack of rain or delayed irrigation) or a hot dry wind cause a substantial reduction in crop performance in terms of either plant survival or economic yield or crop quality. Some of the means to cope with drought by plant have been mentioned. Drought escape is defined as where drought-sensitive stages of plant development (i.e., flowering and fruit seed development) are completed during the part of the season when drought is not present. In contrast, drought resistance is defined as the ability of a cultivar to produce a greater economic yield (or to survive better) than another cultivar, or the ability of a species to be more effective (e.g., profitable) than another species when they are subjected to drought.

For annual cropping in rainfed areas where water is limiting, well adapted cultivars will have an optimal time of flowering and a cycle length from sowing to harvest that fits the patterns of rainfall and water availability. If the cycle is too long, drought during grain filling will reduce seed size and, thereby grain yield. If flowering occurs too early, yield potential will be lower, resulting in smaller grain yields in wet years compared with cultivars that have a longer cycle length.

If economic yield is a reproductive organ, resistance to drought depends on the stage of reproductive development, the type of economic product, and the determinacy of the plant. Plants are often more drought resistant during the vegetative stage than during early flowering or fruit development stages. Plants producing dry grain are more resistant to late-season drought than plants producing fleshy fruit, which require higher turgor. Indeterminate plants, such as cotton, cowpea, and tomatoes, can exhibit superior adaptation to mid-season droughts than determinate plants, such as maize, pearl millet, rice, sorghum, and wheat. This is because, after the droughts, indeterminate plants can produce more leaves, fruits and seeds, whereas determinate plants do not produce any more leaves or seeds on their main stems. Gwathmey and Hall (1992) reported a severe drought during the vegetative stage that reduced the leaf area of the cowpea plants by 74% compared with the weekly irrigated plants, and would have killed most other annual crop species. Determinate crops can be well adapted to late droughts during grain filling, because they can exhibit more complete remobilization of carbohydrate to their grain than the indeterminate crops.

Effects of drought on seed size depend on the separate effects of drought on the relative sizes of the photosynthetic source and the reproductive sink (Fischer, 1980). Seed size has a hyperbolic relationship with the ratio of the photosynthetic source to the reproductive sink. Late drought tends to result in small seed because it

has little influence on the number of seeds produced but strongly reduces the photosynthetic source by accelerating leaf senescence. Drought-induced reduction in seed size tends to be more pronounced with determinate annuals, but they can occur with late drought in indeterminate crops such as cowpea. Mid-season drought can have little effect on seed size in both determinate annuals and indeterminate crops (Turk *et al.*, 1980).

7. Mechanisms of drought resistance

Drought resistance can be considered as depending on the extent of dehydration avoidance, feedforward responses, dehydration tolerance and water use efficiency.

7.1. Dehydration avoidance

Plants can avoid dehydration by maintaining higher leaf water potential (ψ_{leaf}) (closer to zero) when subjected to drought due to having deeper roots accessing moisture present deep in the soil, slower growth of leaf area, and earlier drought-induced stomatal closure compared with other cultivars. However, this adaptation requires a balanced response because all of these mechanisms of dehydration avoidance have costs in terms of processes, causing either greater use or less acquisition of CH_2O . Among plants with substantial resistance to drought, some exhibit little change in ψ_{leaf} (it does not go below -2 MPa) when subjected to drought whereas others exhibit large decreases in ψ_{leaf} down to -5 MPa and a few species can develop very low ψ_{leaf} under extreme drought (Petrie and Hall, 1992).

Plants that develop low values of ψ_{leaf} can still partially maintain RWC if they also adjust osmotically, and experience decreases in osmotic potential (ψ_s) in cells. Plant breeders have developed varieties with greater drought resistance by selecting for greater drought-induced osmotic adjustment in leaves. However, the mechanism may involve osmotic adjustment in roots resulting in the maintenance of pressure potential (ψ_p) in root cells and maintenance of root growth, enabling the improved cultivars to access more soil water than the older cultivars (Ludlow *et al.*, 1983). It should be noted that this adaptation mechanism would be effective only where the deeper roots access substantially more soil water. Some plant species with excellent adaptation to drought exhibit very little drought-induced osmotic adjustment, e.g., cowpea (Petrie and Hall, 1992).

7.2. Feedforward response

The evidence indicates that roots sense critical conditions in the soil and send signals to the shoot that cause partial stomatal closure and slow down leaf expansion before the supply of water or nutrients is affected. Such behavior is known as feedforward, which contrasts with feedback in which it provides advance warning of change. If stomata partially closed or leaf area expansion rate decreased as a feedback

response to a decrease in water status, the plant may suffer damage from the low plant water status. In contrast feedforward mechanisms enable the plant to avoid extreme dehydration. Also, feedback systems can be undesirable in that they have a tendency to exhibit oscillations that represent an inefficient use of resources. Feedforward responses appear to be a component of the sophisticated system that maintains a balance between root and shoot activities.

The significance of feedforward responses to crop production is that they may be too strong and favor plant survival too much over plant productivity and thus be too conservative for annual crop plants in most target production environments. For perennial crops, strong conservative feedforward systems may be desirable. In this case, choice of root stocks may provide opportunities for modifying the root component of the feedforward system in a beneficial manner (Passioura and Stirzaker, 1993).

7.3. Dehydration tolerance

This refers to the extent to which plant function is maintained when relative water content (RWC) decreases. The mechanisms of leaf dehydration tolerance are poorly understood. One hypothesis is that there is a critical RWC at which processes stop or start, such as leaf death. But, higher plants may not respond to damage caused by dehydration. The RWC of plant tissue provides a measure of relative symplast (i.e., cell) volume, and plant may respond to change in volume or more likely to changes in turgor pressure but not the level of dehydration. Ludlow and Muchow (1990) reviewed studies of lethal levels of low water status in plants. They commented that, for pigeon pea subjected to different rates of soil drying, leaf death occurred at a specific critical RWC (32%), irrespective of substantial differences in level of osmotic adjustment and leaf water potential. For pigeon pea, the RWC at which zero turgor occurs is about 80% (Flower and Ludlow, 1986). Thus, the RWC of 32% could represent a leaf that has suffered a catastrophic irreversible inward collapse of cell walls (cyt-orrhysis) resulting from a critical level of negative turgor pressure. An alternative hypothesis is that drought –induced leaf death is really a programmed leaf senescence caused by changes in hormonal signals coming from roots that are being subjected to day-to –day decreases in soil moisture content (Hall, 1993). In this hypothesis, plasma membranes in leaf cells respond to the changes in hormonal signals by permitting osmotica to leave the cells, which would result in an adaptive recycling of nutrients from senescing leaves. The reduction in osmotic pressure (increase in solute potential) within the cells would result in a loss of water, negative turgor, and a collapse of the cells.

Tolerance to dehydration and reduction in activity of water appear to be important in seeds that become very dry at maturity. Certain compounds that accumulate during seed development are thought to play a role in preventing damage to the desiccating embryo. These compounds that include sugars and proteins have been suggested to play a role in desiccation tolerance (Close, 1996).

7.4. Water use efficiency (WUE)

Water use efficiency is the ratio of biomass production to transpiration. Species differences in WUE have been observed. When they are grown in the same optimal warm to hot environment, warm-season C₃ species have much lower WUE than C₄ species, mainly because the C₄ species produce more biomass per day (Hall, *et al.*, 1993). Crop adaptation to drought depends on the additive effects of drought escape and drought resistance. Drought resistance, however, does not depend on the additive effects of dehydration avoidance, feedforward responses, dehydration tolerance and water-use efficiency. Plants with different mechanisms may be equally well adapted to the same semi-arid environment. For example, cereals subjected to soil drought develop low (very negative) leaf water potentials but avoid dehydration by exhibiting osmotic adjustment. Some grain legumes growing in the same environment avoid dehydration by partial stomatal closure and paraheliotropic leaf movements that reduce transpiration (Hall *et al.*, 1993) such that leaf water potential decreases only slightly with soil drought. The possible benefits of the cereal strategy are that, by keeping stomata more open, they may have more photosynthesis, and by developing lower leaf water potentials, they may access slightly more water from the soil. Possible costs of the strategy are associated with osmotica and the construction of tough leaves that can withstand occasional high turgor pressures if de-osmotic adjustment is not sufficiently rapid following rain or sprinkler irrigation. In general, adaptation to drought is conferred by optimal levels of drought escape, dehydration avoidance, feedforward responses, and water-use efficiency in relation to the current and expected types of drought in the specific target production environment.

8. Chitosan application in plants

Chitosan effects on plant response were first characterized as an elicitor. It was shown to be able to activate plant defensive genes through the octadecanoid pathway (Doares *et al.*, 1995). According to the defensive gene induction activity, chitosan was proved to induce disease resistance in several plants, with pathogen and plant cultivar specificity (Eikemo *et al.*, 2003; Bell *et al.*, 1998). Chitosan was also involved in the stomatal response where stomatal opening provides access to inner leaf tissue for plant pathogens, so narrowing stomatal apertures may be advantageous for plant defense. The stomatal apertures of tomato and *Commelina communis* was reduced when the epidermis was treated with chitosan (Lee *et al.*, 1999). It was found in pepper plant that foliar application of chitosan decreased transpiration and reduced water use by 26-43%, while maintained biomass production and yield. Hence, chitosan might be an effective antitranspirant to conserve water use in agriculture (Bittelli *et al.*, 2001). Hirano *et al.* (1990) reported that coating of seeds with depolymerized chitosan or chitosan oligosaccharrides typically increased the chitinase activity in seedlings by 30-50%, unless the seeds had a hard cuticle. Low molecular-weight of chitosan (5 kDa) induced the accumulation of phytoalexins in plant tissue, decreased the total content and changed the composition of free sterols producing adverse effects on infesters, activated chitinase, beta-glucanase, and lipoxygenases and stimulating the generation of reactive oxygen species (Vasiukova *et al.*, 2001). Application of chitin and chitosan to soybean leaf tissues increased activities of

phenylalanine ammonia lyase (PAL) and tyrosine ammonia-lyase (TAL). The elevation of PAL and TAL activity was dependent on the chain length of the oligomers and time after treatment (Wajahatullah *et al.*, 2003). Xianling *et al.* (2002) indicated that seeds of mulberry cultivar Sha 2 were coated with chitosan solution at 3% prepared from silkworm chrysalises increased the respiration rate of germination seeds, root vigor, chlorophyll, protein content and peroxidase in seedlings as well as nitrate reductase and amylase activities. Yue *et al.* (2001) studied maize seeds and reported that chitosan concentration at 2-4 g/litre resulted in a positive effect on endogenous hormone content, alpha-amylase activity and chlorophyll content in seedling leaves. Khan *et al.* (2002) found that on the first day after foliar application, chitosan pentamer (CH5) decreased the net photosynthetic rate of maize, and subsequently there was an increase on day 3 of 10-18% over the control. This increase was related with the increases in stomatal conductance and transpiration rate, while the intercellular CO₂ concentration was not different from the control plants. Chitosan also enhanced the rice endosperm kinase (REK) in RNA expression, which was completely abolished at either 12 or 24 h by cycloheximide (CHX). However, high/low temperature, and the environmental pollutants, ozone and sulfur dioxide, failed to enhance the REK mRNA expression, as determined using in vivo system. These results strongly indicated a function for REK in defense/stress responses in rice photosynthetic tissues (Nam-Soo *et al.*, 2002).

Ohta *et al.* (1999) reported that chitosan application to the soil at sowing time remarkably enhanced plant growth and flowered 15 days earlier than the control. Moreover, a greater number and weight of flowers were produced by chitosan application. Rapeseed (*Brassica chinensis*) c.v. Aikangqin coated with small molecular weight chitosan showed positive effects on germination index, growth of seedlings and root length (Xue *et al.*, 2002). Chibu *et al.* (1999) studied chitosan application on early growth of four crops: soybean, lettuce, tomato and rice. The results showed that chitosan at 0.1 or 0.5% increased leaf area, leaf dry weight and leaf length of soybean, lettuce and rice whereas chitosan at 0.1% showed positive effects on leaf area, leaf length and dry weight of tomato. Seeds of non-heading chinese cabbage cv. Dwarf hybrid No. 1 dressed with chitosan at the rate 0.4-0.6 mg/g of seeds and leaf spraying with 20-40 microgram/ml increased total fresh weight, leaf area, plant height, root length, soluble protein and soluble sucrose in leaves, while the content of crude fiber decreased (Ouyang and Langlai, 2003). Utsunomiya *et al.* (1998) studied the effect of using chitosan oligosaccharides as a soil conditioner with high and low nitrogen on the flowering and fruit growth of purple passion fruit. They found that the numbers of flowers, harvested fruits, fruit weight and juice production were increased significantly by the soil conditioner with high nitrogen. Under conditions of low nitrogen, the soil conditioner reduced flower formation in the first growing season but had little effect on flowering in the next season. This treatment increased fruit and juice weights to nearly the same degree as those observed in the high nitrogen without soil conditioner. Harada *et al.* (1995) reported that application of chitosan in the field increased shoot growth, branch length, node number per plant and seed yield of soybean, and total root length per plant increased by chitosan application in the pot experiment. Krivtsov *et al.* (1996) found that wheat seed c.v. Bezostaya soaked with aqueous solution of polymer (10-

20 kDa) or oligomer (2-4 kDa) at concentration of 50 mg/litre for 3 to 18 hours increased stem and root lengths of seedling by six days after seed treatment. Foliar application at the panicle initiation stage with 0.01 or 0.02% solution of oligochitosan two times (five days apart) increased spike weight, 1,000-grain weight and grain yield by 16%. Ali *et al.* (1997) revealed that the production of nodule fresh weight and nitrogen fixation of soybeans decreased at early growth stage (28 days after sowing, (DAS)) in soil supplemented with chitosan 0.1-0.25%, but increased at the later growth stage (42 DAS). Chibu *et al.* (1999) reported that the top dry weight, leaf area, number of first order thick lateral and second order lateral roots of radish seedlings were increased by chitosan application at 0.1%. Seed of soybeans *c.v.* Junjory soaked with chitosan solution at 1000 ppm for six hours and cultured at 25 degree C for 6 days increased germination percentage, hypocotyl thickness, total length and fresh weight of sprouts by 4, 5, 2 and 1% respectively (Lee *et al.*, 1999). Chitosan has been shown to trigger defense mechanisms in plants (Ryan, 1987). Plants treated with chitin and chitosan produce chitinase that breaks down the chain of chitin and chitosan into more soluble form. They also found that application of various chitin and chitosan oligomers to soybean leaf tissues caused increased activity of phenylalanine ammonia-lyase (PAL) and tyrosine ammonia-lyase (TAL) enzymes. The elevation of enzyme activity was dependent on the chain length of the oligomers and time after treatment (Khan *et al.*, 2003). Nicholson and Hammerschmidt (1992) reported that increases in PAL activity have been demonstrated to be one of the earliest responses of plants to the onset of stress by pathogen infection and are considered as an indication of resistance. Since PAL is the key enzyme in the phenylpropanoid pathway, its activity leads to synthesis of phenols, which are compounds associated with expression of resistance. Loschke *et al.* (1983) reported that chitosan induces the expression of a variety of genes involved in plant defense response that, in some cases, result in increased synthesis of secondary plant metabolites. Chitosan influences pathways involving jasmonic acid (Walker-Simmons *et al.*, 1983; Famer and Ryan, 1990; Doares *et al.*, 1995). Jasmonate exhibits some activities similar to the plant hormone abscisic acid (ABA), which plays a key role in the regulation of water use by plants (Sembdner and Parthier, 1993). Increased levels of ABA result in closure of the plant's stomata and reduced transpiration (Willmer and Pricker, 1996; Leung and Giraudat, 1998). Thus, manipulating the ABA signaling pathway offers the possibility to reduce water consumption by plants (Grill and Ziegler, 1998). Jwa *et al.* (2002) reported that as a fungal elicitor, chitosan enhanced the rice endosperm kinase (REK) mRNA expression, which responds to the critical signals mediating plant self defense/stress response, namely jasmonic acid (JA), salicylic acid (SA) and hydrogen peroxide. Klusener *et al.* (2002) revealed that yeast (*Saccharomyces cerevisiae*) elicitor and chitosan, both elicitors of plant defense response, activated this current activation requiring cytosolic NADPH, and induced elevations in the concentration of free cytosolic calcium ($[Ca]^{2+}_{cyt}$) and stomatal closure in guard cells. Wheat and barley damaged at 2.5 $\mu\text{g/ml}$ of vanadium (in VCl_3) were recovered by treatment with 10-100 $\mu\text{g/ml}$ chitosan irradiated at 700-200 KGy of γ -rays in 1% solution. The reductions of V and Fe contents in plants were due to the ability of chitosan to form chelate complexes with metals in solution. Therefore, it can be concluded that chitosan irradiated at suitable doses (ca. 100 KGy) is effective as a plant growth promoter and heavy metal eliminator in crop production (Tham *et al.*,

2001). Lee *et al.* (1999) reported that chitosan, a component of fungal cell walls, reduced the size of stomatal aperture and inhibited light – induced stomatal opening in tomato epidermis by inducing reactive oxygen species (ROS) such as superoxide and H₂O₂, which inhibit stomatal opening and promote stomatal closing.

MATERIALS AND METHODS

A series of experiments were carried out at Rajamangala University of Technology Suvarnabhumi, Huntra Campus, Pranakhonsriyutthaya Province, during November 2003 to February 2006. There were four sequential experiments, three pot experiments were conducted in a greenhouse and the fourth experiment was done in laboratory. The result of experiment which was previously conducted was used in the next experiment respectively. The details of four experiments are as follows:

Experiment# 1 Effects of molecular weight of chitosan and application method on growth and rice yield

The experimental design was set up in a 3x3 factorial with one control in a randomized complete block design with four replications. Factor A was three application methods: seed soaking, seed soaking+ foliar spraying and foliar spraying. Factor B was 3 chitosan types: monomeric chitosan (degree of deacetylation = 99.74%, molecular weight ~216 KDa), oligomeric chitosan (degree of deacetylation = 95.01%, molecular weight ~10,000 KDa) and polymeric chitosan (degree of deacetylation = 96.62%, molecular weight ~ 100,000 KDa). The control was applied with chemical fertilizer mixed of 46-0-0 and 16-20-0 (ratio:1:1w/w) at the rate of 50 kg/rai without chitosan application. All treatments applied with chemical fertilizer similar to those of control. Chitosan used in this experiment was in form of flake, it was dissolved in 2% dilute acetic acid to be liquid chitosan before applying to the rice plants. Rice seeds c.v. Suphanburi 1 were soaked in chitosan solution for five hours before planting for treatment with seed soaking and soaked in water for the control treatment. Rice seeds were sown in a pot size with 45 cm of diameter containing eight kg/pot of paddy soil, until 14 days after planting, rice seedling was thinned to eight plants per pot. Chitosan at the rate of 20 ppm was sprayed four times: at seedling, tillering, panicle initiation and heading stages. The details of data collection including growth, yield components and grain yield are as follows:

Data Collection

1. Plant height was measured before harvesting time from ten plants per treatment by measuring from the ground to the top of leaves and then averaged to plant height means.
2. Dry matter accumulation was collected at four growth stages (seedling, tillering, panicle initiation and harvesting) from thirty two plants per treatment (four pots) and weighed after drying at 80° C for 48 hours in a hot- air oven.
3. Leaf greenness was measured at seedling, tillering and panicle initiation stages by using chlorophyll meter (SPAD 502). The youngest fully expanded leaves were measured on one side of the midrib of leaf blade, midway between leaf base and

tip. Fifteen readings per treatment were taken as the representation of the SPAD value and averaged to SPAD value means.

4. Tiller numbers were counted at harvesting stage from thirty two plants per treatment (four pots) and averaged to tiller numbers per plant.

5. Seed numbers were counted from ten random panicles after harvesting by separating between filled and unfilled grains and then averaged to filled and unfilled grain numbers per panicle.

6. Thousand- grain weight was weighed base on 14% moisture content by random counting from thousand grains per treatment

7. Grain yield from thirty two plants per treatment (four pots) was measured after harvesting threshing and cleaning and calculated base on 14% moisture content .

Data were subjected to analysis of variance (ANOVA) and least significant difference (LSD) was used to compare treatment mean differences

Experiment # 2 Effects of chitosan concentrations and application frequencies on rice yield

This experiment was conducted with 4×4 factorial with one control in a randomized complete block design with four replications. Factor A was four chitosan concentrations : 20 , 40 , 80 and 100 ppm. Factor B was four foliar applications: one time – at seedling stage; two times – at seedling and tillering stages; three times – at seedling, tillering and panicle initiation stages and four times at seedling, tillering, panicle initiation and heading stages and no chitosan application for control. Polymeric chitosan which showed the best result in experiment #1 was used in this experiment. All treatments were applied with chemical fertilizer mixed of 16-20-0 and 15-15-15 (ratio:1:1w/w) at the rate of 50 kg/rai. Rice seeds were planted in a pot size with 45 cm of diameter containing eight kg/pot of clay soil. Rice seedlings were thinned to eight plants per pot at 14 days after planting. Chitosan was firstly sprayed at seedling stage (20-25 days); the second, third and fourth foliar applications were sprayed at tillering stage (45-50 days), panicle initiation stage (70-75) and heading stage (90 days), respectively with various concentrations. Rice growth, yield components and grain yield were collected as described below:

Data collection

1. Dry matter accumulation was collected from thirty two plants (four pots) per treatment at harvesting stages and weighed after drying at 80° C for 48 hours in a hot- air oven
2. Leaf greenness was measured at panicle initiation stage by using chlorophyll meter (SPAD 502). The youngest fully expanded leaves were measured on one side of the midrib of leaf blade, midway between leaf base and tip. Fifteen readings per treatment were taken as the representation of the SPAD value and averaged to SPAD value means.
3. Tiller numbers were counted at harvesting stage from thirty two plants per treatment (four pots) and averaged to tiller numbers per plant.
4. Panicle numbers were counted from thirty two plants (four pots) per treatment after harvesting and averaged to panicle numbers per plant.
5. Seed numbers were counted from ten random panicles per treatment after harvesting and average to seed numbers per panicle.
6. Thousand- grain weight was weighed base on 14% moisture content by random counting from thousand grains per treatment
7. Grain yield from thirty two plants per treatment (four pots) was measured after harvesting threshing and cleaning and calculated base on 14% moisture content .

All data were subjected to analysis of variance (ANOVA). Least Significant Difference (LSD) was used to determine the difference between the treatment means.

Experiment # 3 Drought recovery and grain yield potential after chitosan application under drought condition

A pot experiment was conducted in a greenhouse. Rice seeds were sown in a pot size with 45 cm of diameter containing eight kg/pot of paddy soil. At 14 days after planting, rice seedlings were thinned to eight plants per pot. The experimental design was a randomized complete block with four replications and five treatments. Chemical fertilizer mixed between 16-20-0 and 15-15-15 at the rate of 50 kg/rai was applied in all treatments. Irrigation was applied in all treatments until 20 days after planting; drought condition was imposed in some treatments by withdrawing irrigation three times: at seedling stage, tillering stage and panicle initiation stage, until rice leaves were tightly rolled, then, re-irrigation was done. Chitosan at 20 ppm concentration was sprayed in some treatments according to treatment details below:

T1 Sufficient irrigation throughout cropping season without chitosan application

T2 At each growth stage, drought period was imposed until rice leaves were tightly rolled (leaf rolling score=9) and then re-irrigation was done without chitosan application (control).

T3 Chitosan was sprayed one day before drought period was imposed, at each growth stage and then re-irrigation was done when rice leaves in control treatment were tightly rolled.

T4 At each growth stage, chitosan was sprayed during drought period (rice leaves were fully cupped (leaf rolling score=5)) and then re-irrigation was done when rice leaves in control treatment were tightly rolled.

T5 At each growth stage, drought period was imposed until rice leaves were tightly rolled and then, re-irrigation was done. One day after re-irrigation chitosan was sprayed to rice leaves.

All data were subjected to analysis of variance (ANOVA). Least Significant Difference (LSD) was used to determine the difference between the treatment means.

Data Collection

Standard evaluation system (SES) (IRRI, 1980) was used to evaluate drought sensitivity (leaf drying, leaf rolling) and drought recovery.

1. Leaf drying was scored at recovery period (7 days after re-irrigation) in each growth stage by using standard evaluation system (SES):

score	Symptoms
0	No symptoms
1	Slight tip-drying
3	Tip drying extended up to $\frac{1}{4}$ length in most leaves
5	$\frac{1}{4}$ to $\frac{1}{2}$ of all leaves fully dried
7	More than $\frac{2}{3}$ of all leaves fully dried
9	All plants apparently dead

2. Leaf rolling was scored during drought stress period in each growth stage by SES:

Score	Symptoms
0	Leaves healthy
1	Leaves starting to fold (shallow V-shape)
3	Leaves folding (deep V-shape)
5	Leaves fully cupped (U-shape)
7	Leaf margins touching (O-shape)
9	Leaves tightly rolled

3. Drought recovery was scored at the same time as leaf drying recording by using SES:

Score	Symptoms
0	No symptoms
1	90% of all plants produce new leaves and tillers after irrigation applied for 1-2 days.
3	75% of all plants produce new leaves and tillers after irrigation applied for 1-2 days
5	75-90% of all plants produce new leaves and tillers after irrigation applied for 4-5 days
7	50-75% of all plants produce new leaves and tillers after irrigation applied for 4-5 days
9	Less than 50% of all plants produce leaves and tillers after irrigation applied for 7 days

4. In each growth stage, plant height was measured from ten plants per treatment after re-irrigation for seven days by measuring from the ground to the top of leaves and averaged to plant height means.

5. Dry matter accumulation was recorded at seedling, tillering and harvesting stages from thirty two plants per treatment and weighed after drying at 80° C for 48 hours in a hot- air oven

6. Leaf greenness was measured at seedling, tillering and panicle initiation stages by using chlorophyll meter (SPAD 502). The youngest fully expanded leaves were measured on one side of the midrib of leaf blade, midway between leaf base and tip. Fifteen readings per treatment were taken as the representation of the SPAD value and averaged to SPAD value means.

7. Tiller numbers were counted at harvesting stage from thirty two plants per treatment (four pots) and averaged to tiller numbers per plant.

8. Panicle numbers were counted from thirty two plants (four pots) per treatment after harvesting and averaged to panicle numbers per plant.

9. Seed numbers were counted from ten random panicles per treatment after harvesting and average to seed numbers per panicle.

7. Grain yield from thirty two plants per treatment (four pots) was measured after harvesting threshing and cleaning and calculated base on 14% moisture content .

All data were subjected to analysis of variance (ANOVA). Least Significant Difference (LSD) was used to determine the difference between the treatment means.

Experiment # 4 Effect of chitosan on physiological responses under drought condition

A pot experiment was conducted in a greenhouse using a randomized complete block design with four treatments and four replications. The details of treatment are as follows:

- T1 Control (no chitosan and fertilizer)
- T2 Chitosan application
- T3 Chemical fertilizer application
- T4 Chitosan + chemical fertilizer application

Rice seeds were sown and thinned to eight plants per pot. Chitosan was sprayed in T2 and T4 at the rate of 20 ppm three times: at 14, 21 and 28 days after planting. Chemical fertilizer mixed of 46-0-0 and 16-20-0 (ratio:1:1w/w) at the rate of 50 kg/rai was applied one times at 14 days after sowing for treatments no. 3 and no.4 . At the end of treatment period (1 month after planting), drought period was imposed by stopping irrigation in all treatments. Rice leaves were taken to assay some physiological changes including nitrate reductase activity (Jaworsaki, 1971) ,soluble sugar (Yoshida,1976),proline accumulation (Bates *et al.*,1973) and total nitrogen content (Janjalernsuk *et al.*, 1998) ,when rice leaves started to fold for early drought period, tightly rolled for the maximum drought period and 7 days after re-irrigation for the recovery period.

All data were subjected to analysis of variance (ANOVA). Least Significant Difference (LSD) was used to determine the difference between the treatment means.

RESULTS AND DISCUSSION

Experiment# 1 Effects of molecular weight of chitosan and application method on growth and rice yield

1. Plant height

Plant height of rice after chitosan application is shown in Table 1. Plant height was not affected by chitosan application. Application with different molecular weights of chitosan and methods did not affect plant height. This result was similar to the report of Khan *et al.* (2002) who reported that foliar application of chitosan oligomer did not affect plant height of soybean. In contrast, Ouyang and Langlai (2003) studied chinese cabbage (*Brassica campestris*) cultivar Dwarf hybrid No.1, found that seed dressing with 0.4-0.6 mg/g seed and leaf spraying 20-40 micro g/ml increased plant height and leaf area of Chinese cabbage.

2. Leaf greenness

Leaf greenness of rice (spad values) at different growth stages are shown in Table 1. The application of chitosan did not significantly affect leaf greenness in all growth stages of rice (seedling, tillering and panicle initiation). Leaf greenness of rice was also not affected by application with different molecular weights of chitosan and methods. This result supported the report of Yue *et al.* (2001) who indicated that the change in chlorophyll content correlated with the change in chitosan concentration, whereas Ouyang and Langlai (2003) found that seeds of non-heading chinese cabbage were dressed with 0.4-0.6 mg/g seeds and leaf spraying with 20-40 micro g/ml increased in chlorophyll content. Moreover, Chibu and Shibayama (1999) reported that leaves of tomatoes and lettuces became darker green as the chitosan concentration increased.

3. Dry matter accumulation

Table 2 shows dry matter accumulation of rice as affected by chitosan application. Application of chitosan did not affect dry matter accumulation at seedling and tillering stage. At panicle initiation stage, dry matter accumulation from treatment with polymeric chitosan by seed soaking followed by foliar sprays significantly increased over the control. At harvesting stage, dry matter accumulation was affected by chitosan application with different molecular weights, polymeric chitosan showed the best effect. There are many reports that supported this result such as Ali *et al.* (1997) who revealed that dry matter accumulation of soybean cultivar Akishirome was increased at 42 days after sowing with soils supplemented with 0.1 chitosan. Ouyang and Langlai (2003) also reported that seeds of non-heading chinese cabbage dressed with chitosan at the rate 0.4-0.6 mg/g seed and leaf spraying with 20-40 micro g/ml increased fresh weight. The result of Hidalgo *et al.* (1996) showed that tomato

plants grown from seed coated with chitosan increased dry weight and stem thickness more than untreated plants.

4. Filled and unfilled grain numbers

Table 3 show numbers of filled and unfilled grains of rice after chitosan application with different molecular weights and methods. The unfilled grain numbers of rice was significantly different after chitosan application. Application with polymeric chitosan significantly obtained unfilled grain numbers lower than the other treatments and the control. Different application methods did not affect unfilled grain numbers, however, application with polymeric chitosan by seed soaking before planting and followed by foliar sprays significantly reduced unfilled grain when compared with the others. In contrast, filled grain numbers were not affected by chitosan application. Rice plant applied with chitosan was rather darker green because chitosan molecules contained many nitrogen atoms which may contribute to leaf greenness. Therefore, there was higher potential for rice applied with chitosan to produce greater photosynthate. As a consequence, food reserve in vegetative part was higher, and potentially was translocated to fill more grain. This result supported the work of Utsunomiya *et al.* (1998) who found that the numbers of flowers and harvested fruits of purple passion fruit were increased with soil treated with chitosan oligosaccharides under high nitrogen conditions.

5. Thousand - grain weight

Thousand grain weights of rice are given in Table 3. Application of chitosan did not show any significant differences on thousand grain weight when compared with control. Chitosan application with different molecular weights and methods did not increase thousand grain weight. This result is contrary to Krivtsov *et al.* (1996) who found that thousand grain weight and spike weight of wheat plants were increased with treatment with low concentrations of polymeric chitosan.

6. Tiller numbers per plant

The effect of chitosan with different molecular weights on the tiller numbers per plant was given in Table 4. Tiller numbers of rice were affected by different types of chitosan. Application with polymeric chitosan showed significantly increased in tiller numbers per plant but methods of application did not affect tiller numbers. Soaking the seeds with polymeric chitosan followed by foliar spray tended to increase tiller numbers per plant. A similar result was also reported by Harada *et al.* (1995) who showed that soil application with chitosan increased branch and node numbers per plant of soybean. Similarly, Lu *et al.* (2002) found that number of panicles of rice increased with rice seeds sown and watered with 0.4 g chitosan/50 cm³ water. Ohta *et al.* (2001) also reported that the application of soil mixed with chitosan 1% w/w at sowing remarkably increased number of flowers of *Eustoma grandiflorum*.

7. Grain yield

The effects of different molecular weights and methods of application on rice yield are shown in Table 4. Application of chitosan showed significant differences on yield when compared with control. Rice yield was not affected by application of chitosan with different molecular weights and methods. However, the average of grain yield obtained from soaking seed before planting and followed by foliar sprays later with polymeric chitosan (170.2 g/trt) tended to be higher than those of other treatments and the control (111.7g/trt). The chain of polymeric chitosan was long and comprised many glucosamine units containing nitrogen compounds which contributed to plant growth and yield potential. This result supported the report of Krivtsov *et al.*,(1996) who found that wheat plants treated with polymeric or oligomeric chitosan increased spike weight and grain yield.

Table 1 Effects of chitosan with different molecular weights and application method on leaf greenness at various growth stages and plant height.

Treatment	Seedling stage (SPAD unit)	Tillering stage (SPAD unit)	Panicle initiation stage (SPAD unit)	Plant height (cm)
Control	39.95	36.70	36.62	95.75
Treatments	40.25	36.56	36.86	98.82
Main plot (M)				
Seed soaking	40.40	36.78	36.38	97.87
Seed soaking + Foliar spray	40.65	36.05	37.10	98.14
Foliar spray	39.70	36.85	37.10	100.45
Sub plot (S)				
Monomer	39.77	36.62	36.16	98.50
Oligomer	40.49	36.60	36.90	99.20
Polymer	40.50	36.46	37.52	98.77
Main plot x Sub plot (MxS)				
Seed soaking x Monomer	40.45	36.52	35.67	99.75
Seed soaking x Oligomer	40.42	36.72	36.12	96.81
Seed soaking x Polymer	40.35	37.10	37.35	97.06
Seed soaking + Foliar spray x Monomer	40.47	35.70	36.05	96.56
Seed soaking + Foliar spray x Oligomer	41.05	36.12	37.22	99.43
Seed soaking + Foliar spray x Polymer	40.42	36.35	38.05	98.43
Foliar spray x Monomer	38.40	37.65	36.77	99.18
Foliar spray x Oligomer	40.00	36.95	37.37	101.37
Foliar spray x Polymer	40.72	35.95	37.17	100.81
LSD.05 Control x Treatment	ns	ns	ns	ns
LSD.05 Main plot	ns	ns	ns	ns
LSD.05 Sub plot	ns	ns	ns	ns
LSD.05 Main plot x Sub plot	ns	ns	ns	ns
CV. (%)	2.75	3.98	4.17	2.78

Table 2 Effects of chitosan with different molecular weights and application method on dry matter accumulation per treatment at various growth stages.

Treatment	Seedling stage (g)	Tillering stage (g)	Panicle initiation stages (g)	Harvesting stages (g)
<u>Control</u>	27.20	219.52	303.36	595.20
<u>Treatments</u>	28.48	271.36	496.00	710.00
Main plot (M)				
Seed soaking	28.16	263.36	476.16 b	725.76
Seed soaking + Foliar spray	30.40	273.92	583.04 a	739.84
Foliar spray	27.20	277.12	429.12 b	665.60
Sub plot (S)				
Monomer	26.56	260.48	475.20	689.92b
Oligomer	29.44	264.32	472.96	678.08b
Polymer	29.76	289.06	536.00	763.20a
Main plot x Sub plot (MxS)				
Seed soaking x Monomer	27.20	246.08	506.88 bc	720.00
Seed soaking x Oligomer	28.48	268.48	406.08 c	674.88
Seed soaking x Polymer	28.48	275.52	515.52 bc	782.40
Seed soaking + Foliar spray x Monomer	28.16	251.84	430.72 c	686.72
Seed soaking + Foliar spray x Oligomer	31.36	252.80	631.68 ab	727.36
Seed soaking + Foliar spray x Polymer	31.68	317.44	687.36 a	804.80
Foliar spray x Monomer	24.64	283.20	500.48 bc	662.40
Foliar spray x Oligomer	28.16	272.32	380.80c	632.32
Foliar spray x Polymer	29.12	275.84	405.44c	702.40
LSD.05 Control x Treatment	ns	ns	*	*
LSD.05 Main plot	ns	ns	50.88	ns
LSD.05 Sub plot	ns	ns	ns	64.96
LSD.05 Main plot x Sub plot	ns	ns	164.8	ns
CV. (%)	13.91	24.98	22.77	10.48

Table 3 Effects of chitosan with different molecular weights and application method on filled grain numbers, unfilled grain numbers and thousand grain weight.

Treatment	Filled grain numbers per panicle	Unfilled grain numbers per panicle	Thousand grain Weight (g)
<u>Control</u>	73.75	32.40	26.47
<u>Treatments</u>	78.73	22.67	25.76
Main plot (M)			
Seed soaking	72.15	23.06	25.59
Seed soaking + Foliar spray	87.05	22.03	25.89
Foliar spray	77.0	22.93	25.81
Sub plot (S)			
Monomer	77.13	25.86 a	25.41
Oligomer	76.24	22.08 ab	26.37
Polymer	82.80	20.08 b	25.50
Main plot x Sub plot (MxS)			
Seed soaking x Monomer	71.75	24.25 ab	25.55
Seed soaking x Oligomer	69.72	22.55 ab	24.57
Seed soaking x Polymer	74.90	22.40 ab	26.65
Seed soaking + Foliar spray x Monomer	80.35	28.75 a	25.32
Seed soaking + Foliar spray x Oligomer	87.85	23.05 ab	26.90
Seed soaking + Foliar spray x Polymer	92.95	14.30 c	25.45
Foliar spray x Monomer	79.30	24.60 ab	25.37
Foliar spray x Oligomer	71.15	20.65 bc	27.65
Foliar spray x Polymer	80.55	23.55 ab	24.42
LSD.01 Control x Treatment	ns	*	ns
LSD.05 Main plot	ns	ns	ns
LSD.05 Sub plot	ns	3.82	ns
LSD.05 Main plot x Sub plot	ns	6.62	ns
CV. (%)			

Table 4 Effects of chitosan with different molecular weights and application method on grain yield and tiller numbers .

Treatment	Grain yield per treatment (g)	Tiller numbers per plant (tiller)
<u>Control</u>	111.72	4.42
<u>Treatments</u>	149.31	6.64
Main plot (M)		
Seed soaking	134.68	6.25
Seed soaking + Foliar spray	160.75	6.90
Foliar spray	152.50	6.77
Sub plot (S)		
Monomer	140.35	6.46 b
Oligomer	150.48	6.36 b
Polymer	157.10	7.09 a
Main plot x Sub plot (MxS)		
Seed soaking x Monomer	131.02	5.82
Seed soaking x Oligomer	141.56	6.25
Seed soaking x Polymer	131.47	6.67
Seed soaking + Foliar spray x Monomer	148.26	6.95
Seed soaking + Foliar spray x Oligomer	162.73	6.02
Seed soaking + Foliar spray x Polymer	171.25	7.72
Foliar spray x Monomer	141.76	6.62
Foliar spray x Oligomer	147.16	6.82
Foliar spray x Polymer	168.59	6.87
LSD.05 Control x Treatment	*	*
LSD.05 Main plot	ns	ns
LSD.05 Sub plot	ns	0.53
LSD.05 Main plot x Sub plot	ns	ns
CV. (%)	20.71	9.58

Experiment # 2 Effects of chitosan concentrations and application frequencies on rice yield

1. Leaf greenness

Leaf greenness of rice was not affected by chitosan application. The result of leaf greenness is shown in Table 5. Leaf greenness did not show any significant differences when applied with various chitosan concentrations. Foliar application frequency did not affect leaf greenness. This result was similar to the work of Yue *et al.* (2001) who found that the changing regulation of chlorophyll content was correlated with the change in chitosan concentration. Moreover, XianLing *et al.* (2002) showed that the chlorophyll content of mulberry cultivar Sha 2 increased with increasing chitosan concentration prepared from silkworm chrysalises.

2. Dry matter accumulation

Dry matter accumulation was affected by chitosan application. The result of dry matter accumulation is shown in Table 5. Four foliar applications significantly affected dry matter accumulation. Dry matter accumulation was not affected by application with various chitosan concentrations, However, application at the rate of 20 ppm tended to accumulate dry matter over the control. This result was supported by Chibu and Shibayama (1999) who revealed that dry weight of dry- land rice cultivar Misatohatamochi grown with both 0.1 and 0.5% of chitosan were increased over the control. Furthermore, Brodelius *et al.* (1989) also reported that maximum product formations of suspension cultures of *Nicotiana tobaccum* and *Eschscholtzia Californica* were observed from cells elicited with the highest chitosan concentration

3. Thousand -grain weight

Thousand-grain weight is shown in Table 6. It was not affected by chitosan application. Different concentrations did not significantly affect thousand grain weight. Thousand grain weight was not affected by various application frequencies. This result contradicted to the work of Krivtsov *et al.* (1996) who found that sprinkling wheat plants at the ear formation stage with 0.01 or 0.02% solution of chitosan increased thousand -grain weight.

4. Seed numbers per panicle

Seed numbers per panicle are presented in Table 6. Chitosan application did not affect seed numbers per panicle. Four foliar applications tended to gain the highest seed numbers per panicle over the others, but did not show any significant differences when compared with the others and the control. Seed numbers per panicle were not affected by various chitosan concentrations.

5. Tiller numbers per plant

The results in Table 7 show tiller numbers per plant after chitosan application. Chitosan application did not show any significant differences on tiller numbers of rice. Application with different concentration did not significantly affect tiller numbers per plant. However, chitosan application at 20 ppm tended to give higher tiller numbers per plant (5.0 tillers). Four foliar applications tended to get higher tiller numbers per plant (5.0 tillers) but did not show any significant differences among treatments as well as the control.

6. Panicle numbers per plant

The panicle numbers per plant are presented in Table 7. The panicle numbers were greatly affected by chitosan application. Four foliar applications significantly increased panicle numbers per plant over the other treatments. Panicle numbers per plant were not affected by various chitosan concentrations, however there was a tendency to get higher panicle numbers at the concentration of 20 ppm. This result supported the report of Lu *et al.* (2002) who revealed that rice seeds sown and watered with 0.4 g chitosan / 50 cm³ showed higher panicle numbers than the control.

7. Grain yield

Grain yield after chitosan application is presented in Table 7. Chitosan application did not affect grain yield. Application of chitosan at various concentrations did not significantly affect rice yield. However, application at 20 ppm of concentration tended to obtain higher grain yield than the other concentrations. Grain yield was not significantly increased with various application frequencies. The mean grain yield from treatment with four foliar application with 20 ppm of concentration tended to be the highest (220g/trt) but did not show significant differences. Different species differently responded to chitosan concentration. The optimum concentration for one plant species may not be suitable for another plant species. (Yue *et al.*, 2001). Some plant species responded with high concentration such as in the report of Lee *et al.* (1999) who found that the optimum chitosan concentration for soybean cultivar Junjory was 1,000 ppm. This result was similar to the report of Hong *et al.* (1998) who showed that yield of indica rice is 17.6% above control after being treated with dilute chitosan in the range of 500 times. A similar result was also reported by Krivtsov *et al.* (1996) who found that wheat plants sprinkled at the ear formation stage with 0.01 or 0.02% solution of chitosan increased grain yield by 16%.

Table 5 Effects of chitosan concentrations and application frequencies on dry matter accumulation and leaf greenness.

Treatment	Dry matter accumulation per treatment (g)	Leaf greenness (SPAD unit)
Control	538.4	36.36
Treatments	625.6	37.6
Main plot (M)		
Concentration at 20 ppm	656.0	37.8
Concentration at 40 ppm	603.2	37.5
Concentration at 80 ppm	644.8	37.6
Concentration at 100 ppm	603.2	37.5
Sub plot (S)		
One foliar spray	571.2 b	37.1
Two foliar sprays	616.0 ab	38.2
Three foliar sprays	630.4 ab	37.0
Four foliar sprays	688.0 a	38.1
Main plot x Sub plot (MxS)		
Concentration at 20 ppm x One foliar spray	620.8	38.1
Concentration at 20 ppm x Two foliar sprays	625.6	37.7
Concentration at 20 ppm x Three foliar sprays	667.2	38.6
Concentration at 20 ppm x Four foliar sprays	712.0	37.0
Concentration at 40 ppm x One foliar spray	491.2	36.1
Concentration at 40 ppm x Two foliar sprays	678.4	37.9
Concentration at 40 ppm.x Three foliar sprays	532.8	36.7
Concentration at 40 ppm.x Four foliar sprays	708.8	39.5
Concentration at 80 ppm. x One foliar spray	627.2	37.2
Concentration at 80 ppm. x Two foliar sprays	603.2	39.1
Concentration at 80 ppm x Three foliar sprays	673.6	36.3
Concentration at 80 ppm x Four foliar sprays	673.6	37.7
Concentration at 100 ppm x One foliar spray	546.6	36.9
Concentration at 100 ppm x Two foliar sprays	558.4	38.3
Concentration at 100 ppm.x Three foliar sprays	649.6	36.7
Concentration at 100 ppm.x Four foliar sprays	656.0	38.3
LSD.05 Control x Treatment	ns	ns
LSD.05 Main plot (M)	ns	ns
LSD.05 Sub plot (S)	75.35	ns
LSD.05 Main plot x Sub plot (MxS)	ns	ns
CV(%)	14.25	5.5

Table 6 Effects of chitosan concentrations and application frequencies on thousand-grain weights and seed numbers.

Treatment	Thousand grain weights (g)	Seed numbers per panicle
<u>Control</u>	23.6	88.3
<u>Treatments</u>	25.7	98.9
Main plot (M)		
Concentration at 20 ppm	26.3	99.5
Concentration at 40 ppm.	24.9	99.5
Concentration at 80 ppm.	26.5	100.5
Concentration at 100 ppm.	25.2	96.4
Sub plot (S)		
One foliar spray	24.9	99.5
Two foliar sprays	26.0	96.4
Three foliar sprays	26.0	96.8
Four foliar sprays	26.2	103.0
Main plot x Sub plot (MxS)		
Concentration at 20 ppm x One foliar spray	25.3	97.3
Concentration at 20 ppm x Two foliar sprays	26.6	100.0
Concentration at 20 ppm x Three foliar sprays	26.4	102.6
Concentration at 20 ppm x Four foliar sprays	26.8	98.0
Concentration at 40 ppm. x One foliar spray	24.6	97.6
Concentration at 40 ppm. x Two foliar sprays	24.4	100.0
Concentration at 40 ppm.x Three foliar sprays	24.4	94.0
Concentration at 40 ppm.x Four foliar sprays	25.9	106.6
Concentration at 80 ppm. x One foliar spray	25.5	107.0
Concentration at 80 ppm. x Two foliar sprays	26.8	95.6
Concentration at 80 ppm.x Three foliar sprays	26.8	95.6
Concentration at 80 ppm.x Four foliar sprays	26.9	104.0
Concentration at 100 ppm x One foliar spray	24.5	96.3
Concentration at 100 ppm. x Two foliar sprays	26.2	90.3
Concentration at 100 ppm.x Three foliar sprays	26.2	95.3
Concentration at 100 ppm.x Four foliar sprays	25.2	103.6
LSD.05 Control x Treatment	ns	ns
LSD.05 Main plot (M)	ns	ns
LSD.05 Sub plot (S)	ns	ns
LSD.05 Main plot x Sub plot (MxS)	ns	ns
CV. (%)	7.57	11.08

Table 7 Effects of chitosan concentrations and application frequencies on grain yield, tiller numbers and panicle numbers.

Treatment	Rice yield per treatment (g)	Tiller numbers per plant	Panicle numbers per plant
<u>Control</u>	166.40	4.3	2.7
<u>Treatments</u>	168.80	4.7	3.2
Main plot (M)			
Concentration at 20 ppm	176.24	5.0	3.6
Concentration at 40 ppm.	161.60	4.6	3.2
Concentration at 80 ppm.	168.00	4.5	3.1
Concentration at 100 ppm.	169.60	4.6	2.7
Sub plot (S)			
One foliar spray	152.48	4.2	2.5 b
Two foliar sprays	163.44	4.6	3.4 a
Three foliar sprays	174.48	5.0	3.3 a
Four foliar sprays	185.60	5.0	3.5 a
Main plot x Sub plot (MxS)			
Concentration at 20 ppm x One foliar spray	158.96	4.5	2.7
Concentration at 20 ppm x Two foliar sprays	164.96	5.3	3.8
Concentration at 20 ppm x Three foliar sprays	192.96	5.5	4.3
Concentration at 20 ppm x Four foliar sprays	188.32	4.8	3.6
Concentration at 40 ppm. x One foliar spray	166.16	4.2	2.7
Concentration at 40 ppm. x Two foliar sprays	153.60	4.2	3.2
Concentration at 40 ppm.x Three foliar sprays	144.64	5.4	3.4
Concentration at 40 ppm.x Four foliar sprays	182.16	4.6	3.7
Concentration at 80 ppm. x One foliar spray	134.16	4.1	2.6
Concentration at 80 ppm. x Two foliar sprays	173.60	4.5	3.6
Concentration at 80 ppm.x Three foliar sprays	180.80	4.7	2.6
Concentration at 80 ppm.x Four foliar sprays	184.00	4.6	3.7
Concentration at 100 ppm x One foliar spray	150.64	4.0	2.0
Concentration at 100 ppm. x Two foliar sprays	161.20	4.2	3.0
Concentration at 100 ppm.x Three foliar sprays	179.52	4.3	2.8
Concentration at 100 ppm.x Four foliar sprays	188.00	5.9	2.9
LSD.05 Control x Treatment	ns	ns	ns
LSD.05 Main plot (M)	ns	ns	ns
LSD.05 Sub plot (S)	ns	ns	0.526
LSD.05 Main plot x Sub plot (MxS)	ns	ns	ns
CV. (%)	16.88	21.54	21.59

Experiment # 3 Drought recovery and grain yield potential after chitosan application under drought condition

1. Leaf rolling

Table 8 shows leaf rolling score after chitosan application under drought condition. Leaf rolling showed highly significant differences among treatments with and without chitosan application under drought condition. Leaf rolling of treatment applied with chitosan before drought (6.08 score) was less than the others in all growth stages (9, 8.16 and 7.8 scores). The severe leaf rolling (9 score) was observed in treatment without chitosan under drought. It was found that at the same time, while rice leaves under drought without chitosan application were tightly rolled, rice leaves applied with chitosan before drought were only fully cupped (U-shape).

2. Leaf drying

Leaf drying after chitosan application under drought is shown in Table 9. Chitosan application significantly affected leaf drying. Severe leaf drying (4.53 score) of rice was observed in the treatment without chitosan application. Chitosan application before drought significantly decreased leaf drying (2.04 score) when compared with the other treatments (4.53, 3.87 and 3.27 scores). However, leaf drying was not significantly different between treatments applied with chitosan during and after drought.

3. Drought recovery

Drought recovery of rice plants is shown in Table 10. It was affected by chitosan application. Drought recovery in the treatment applied with chitosan before drought was significantly different from treatment without chitosan application. Rice plants applied with chitosan before drought were recovered faster than those of the others after re-irrigation, but drought recovery between treatments applied with chitosan during and after drought was not significantly different whereas treatment without chitosan showed less drought recovery than the others. Considering throughout the cropping season, it was found that about 68-83% of rice plants from treatment without chitosan application recovered after re-irrigation for 4-5 days, while 81% of those applied with chitosan before drought recovered after re-irrigation for 1-2 days.

4. Dry matter accumulation

The dry matter accumulation of rice plants at various growth stages after chitosan application is shown in Table 11. Dry matter accumulation was significantly different between treatments under drought and sufficient irrigation. Under drought condition, chitosan application did not affect dry matter accumulation in all growth stages. However, chitosan application before drought tended to accumulate higher dry

matter than those in the other treatments. The accumulation of dry matter in treatment without chitosan was the lowest in all growth stages.

5. Plant height

The height of rice plants at different growth stages is shown in Table 12. Plant height at seedling stage was significantly different between treatments under drought and sufficient irrigation. Under drought condition, plant height was not affected by chitosan application. Application of chitosan before, during and after drought was not significantly different on plant height.

6. Leaf greenness

Leaf greenness of rice plants at different growth stages is shown in Table 13. Leaf greenness was significantly different between treatments under drought and sufficient irrigation in tillering stage but no significant difference in seedling and panicle initiation stages. However, under drought, chitosan application did not affect leaf greenness of rice plant in all growth stages.

7. Yield components

Rice yield components; tiller numbers per plant, panicle numbers per plant and seed numbers per panicle are shown in Table 14. Tiller numbers per plant were significantly affected by chitosan application. Tiller numbers per plant in treatment applied chitosan before drought (4.4 tillers) were over the others which applied chitosan during, after drought and control (3.9, 57 and 2.52 tillers). Chitosan application significantly affected panicle numbers per plant. The maximum panicle numbers per plant were obtained from treatment applied with chitosan before drought (3.1 panicles). The panicle numbers per plant in treatment applied with chitosan during, after drought and control were 2.0, 1.97 and 1.57 tillers respectively. Seed numbers per panicle were significantly different between treatments applied and not applied with chitosan. Chitosan application before drought showed higher seed numbers per panicle (88.75 seeds) than those in treatment applied with chitosan during, after drought and control (78.5, 72.5 and 65.75 seeds).

8. Yield

Rice yield was affected by chitosan application and the result is shown in Table 14. Rice yield was significantly affected by chitosan application. Grain yield obtained from treatment applied with chitosan before drought (133.0 g/trt.) was higher than those in other treatments applied with chitosan during (96.0g/trt), after drought (96.75g/trt) and control (74.75 g/trt). It could be explained that all physiological mechanism processes in rice plants in treatment applied with chitosan before drought were still functional to some degree. For the treatment that chitosan was applied before drought, chitosan could be absorbed and changed into available form by some

enzymes resulting in triggering of the defense mechanisms by stomata closing or phytoalexin production resulting in maintaining growth and yield (Bittelli *et al.*, 2001; Walker – Simmons *et al.*, 1983).

Table 8 Effect of chitosan on leaf rolling score under drought condition.

Treatment	Leaf rolling score ^{a/} (score)			
	Seedling stage	Tillering Stage	Panicle Initiation stage	Average
Sufficient Irrigation	0 a	0 a	0 a	0
Drought without chitosan (control)	9 c	9 c	9 d	9
Chitosan applied before drought	6.75 b	6 b	5.5 b	6.08
Chitosan applied during drought	9 c	8 c	7.5 c	8.16
Chitosan applied after drought	9 c	7.5 bc	7 c	7.8
LSD.01	1.14	1.67	1.42	
(%) C.V.	11.17	12.70	11.35	

a/ = Leaf rolling score by SES (IRRI,1980)

0 = Leaves healthy

1 = Leaves starting to fold (shallow V-shape)

3 = Leaves folding (deep V- shape)

5 = Leaves fully cupped (U-shape)

7 = Leaf margins touching (O-shape)

9 = Leaves tightly rolled

Table 9 Effect of chitosan on leaf drying score under drought condition.

Treatment	Leaf drying score (score) ^{b/}			
	Seedling Stage	Tillering stage	Panicle initiation stage	Average
Sufficient irrigation	0 a	0 a	0 a	0
Drought without chitosan	2.25 c	4.95 c	6.41 d	4.53
Chitosan applied before drought	1.12 b	2.88 b	2.13 b	2.04
Chitosan applied during drought	1.35 cb	4.68 c	5.6 cd	3.87
Chitosan applied after drought	1.57 cb	3.38 bc	4.88 c	3.27
LSD.01	0.92	1.76	1.503	
(%) C.V.	23.88	23.59	18.52	

b/ = Leaf drying score by SES (IRRI,1980)

0 = No symptoms

1 = Slight tip drying

3 = Tip drying extended up to ¼ length in most leaves

5 = ¼ to ½ of all leaves fully dried

7 = More than 2/3 of all leaves fully dried

9 = All plants apparently dead

Table 10 Effect of chitosan on drought recovery score under drought condition.

Treatment	Drought recovery score ^{c/} (score)			
	Seedling stage	Tillering stage	Panicle initiation stage	Average
Sufficient irrigation	0 a	0 a	0 a	0
Drought without chitosan	3 b	7 d	8 d	6
Chitosan applied before drought	1 a	2 b	5.5 b	2.8
Chitosan applied during drought	1.5 ab	5.5 c	7 cd	4.6
Chitosan applied after drought	2.5 b	6 cd	6.5 bc	5
LSD.01	1.892	1.476	1.421	
(%) C.V.	24.72	16.66	10.62	

c/= Drought recovery score by SES (IRRI,1980)

0 = No symptoms

1 = 90 % of all plants produce new leaves and tillers after re- irrigation for 1-2 days

3 = 75 % of all plants produce new leaves and tillers after re-irrigation for 1-2 days

5 = 75-90% of all plants produce new leaves and tillers after re- irrigation for 4-5days

7 = 50-75% of all plants produce new leaves and tillers after re-irrigation for 4-5 days

9 = Less than 50% of all plants produce new leaves and tillers after re-irrigation for 7 days

Table 11 Effect of chitosan on dry matter accumulation under drought condition.

Treatment	Dry matter accumulation (g)		
	Seedling stage	Tillering stage	Panicle initiation stage
Sufficient irrigation	53.45	181.08 a	432.0 a
Drought without chitosan	41.87	108.04 b	151.84 b
Chitosan applied before drought	45.07	138.0 b	236.4 b
Chitosan applied during drought	48.00	112.8 b	199.38 b
Chitosan applied after drought	49.65	112.0 b	207.96 b
LSD.01	ns	40.78	86.3
(%) C.V.	15.98	14.80	16.27

Table 12 Effect of chitosan on plant height under drought condition.

Treatment	Plant height (cm)		
	Seedling stage	Tillering stage	Panicle Initiation Stage
Sufficient irrigation	86.3 a	99.25	118.5
Drought without chitosan	78.25 bc	89.25	112.25
Chitosan applied before drought	82.0 ab	91.0	121.75
Chitosan applied during drought	82.75 ab	90	114.0
Chitosan applied after drought	78.25 bc	89.75	119.0
LSD. 05	4.719	ns	ns
CV (%)	3.76	13.16	7.87

Table 13 Effect of chitosan on leaf greenness under drought condition.

Treatment	Leaf greenness (SPAD unit)		
	Seedling stage	Tillering stage	Panicle initiation stage
Sufficient irrigation	39.9	39.71 a	32.73
Drought without chitosan	37.5	37.84 b	31.06
Chitosan applied before drought	38.56	38.95 ab	31.80
Chitosan applied during drought	37.93	38.80 ab	30.73
Chitosan applied after drought	37.16	37.73 b	31.80
LSD.05	ns	1.464	ns
(%) C.V.	4.45	8.67	5.5

Table 14 Effect of chitosan on grain yield and yield components under drought condition.

Treatment	Grain yield per treatment (g)	Tiller numbers per plant	Panicle numbers per plant	Seed numbers per panicle
Sufficient irrigation	196.5 a	4.62 a	3.52 a	111.5 a
Drought without chitosan	74.75 c	2.52 c	1.57 c	65.75 c
Chitosan applied before drought	133.0 b	4.4 ab	3.1 ab	88.75 b
Chitosan applied during drought	96.0 c	3.9 ab	2.0b c	78.5 bc
Chitosan applied after drought	96.75 c	3.57 b	1.97 bc	72.5 bc
LSD.01	24.39	0.898	1.189	17.27
(%) C.V.	9.46	10.94	22.61	9.59

Experiment # 4 Effect of chitosan on physiological responses under drought condition

1. Nitrate Reductase Activity

The activity of nitrate reductase (NR) of rice seedlings was not affected by chitosan application in the early drought period. However, the maximum activities tended to be detected from the treatment applied with chitosan and fertilizer but there were no significant differences among treatments. In the severe drought period, the activity of nitrate reductase was significantly affected by fertilizer application. Treatments without fertilizer application showed higher activity of nitrate reductase than those with fertilizer application (Table 15). It can be demonstrated that the growth and development of rice plants in treatment without fertilizer application were rather poor, resulting in less water use, but the volume of water applied in each treatment was equal. Therefore, the moisture remainder in soil of these treatments were still enough for plant to continue some physiological metabolism associated with the activity of nitrate reductase whereas the growth and development of rice plants in treatments with fertilizer application were somewhat rapid and demanded a lot of water to maintain some physiological metabolism processes, so that, when they were in severe drought condition, some metabolic processes were suddenly interrupted resulting in reduction of nitrate reductase activity. In terms of plant recovery, it was found that the activity of NR in treatments without fertilizer application slowly increased after 7 days of re-irrigation but the increase in NR activity was detected in treatments applied with chitosan and fertilizer. This study is supported by many reports such as work of Shen (1972) who found that nitrate reductase activities were induced by organic compounds containing the nitro – group in the molecules because NR activity of most higher plant species requires NADH as electron donor (Evans and Nason, 1953). Chitosan is a biopolysaccharide containing many nitro – groups in its molecules. It may contribute to increase the activity of NR. Maria *et al.* (2005) revealed that nitrate reductase activities were negatively affected by soil drying. A similar result has been reported by Diouf *et al.* (2004) who found that the increases in nitrogen concentration under water deficit condition showed a reduction in NR activity.

Table 15 Effect of chitosan on nitrate reductase activities under drought condition. (μg nitrite/g leaf fresh weight).

Treatment	Early drought period	Severe drought period	Recovery period
Control	0.113	0.116 a	0.157
Chitosan application	0.152	0.115 a	0.158
Fertilizer application	0.166	0.076 b	0.192
Chitosan+ fertilizer application	0.171	0.089 ab	0.221
LSD.01	ns	ns	ns
LSD.05	ns	0.031	ns
C.V.(%)	25.79	21.08	11.40

2. Proline accumulation

In the early drought period, no significant difference in proline accumulation was detected among treatments. But in the severe drought period, there were highly significant differences between treatments applied and not applied with chitosan under the same rate of fertilizer application. The levels of proline accumulation obtained from treatment applied with chitosan and fertilizer were higher than those of the others. In the recovery period, the proline levels continuously decreased in all treatments and were still significantly different between treatments with and without fertilizer application (Table 16). This finding may be explained by the ability of proline accumulation in rice plants applied with chitosan and fertilizer being better than those of the other treatments due to the additive effects between fertilizer and chitosan which triggers defense mechanism in plants against abiotic and biotic stresses (Khan *et al.*,2002). This result was similar to the work of Choudhary *et al.* (1996) who reported that proline accumulation of *Ziziphus rotundifolia* seedlings under moisture stress may contribute to osmotic adjustment resulting in drought tolerance. Therefore, high proline concentrations may help protecting cell metabolism and facilitating recovery after drought stress (Rhodes and Handa,1989).

Table 16 Effect of chitosan on proline accumulation under drought condition. (μg proline/g fresh weight).

Treatment	Early drought period	Severe drought period	Recovery period
Control	89.79	1383.14 c	165.28 b
Chitosan application	92.19	1269.83 c	236.78 ab
fertilizer application	95.54	29574.18 b	280.82 ab
Chitosan+fertilizer application	94.58	32817.05 a	349.13 a
LSD.01	ns	2369	118.7
LSD.05	ns	1649	82.64
C.V.(%)	8.20	6.34	19.64

3. Soluble sugar accumulation

The soluble sugar accumulation in rice plants was not significantly different among treatments in the early drought period. In the severe drought period, the soluble sugar accumulation was significantly different between treatments applied and not applied with fertilizer. Treatment applied with chitosan and fertilizer showed the highest soluble sugar accumulation. In the recovery period, the soluble sugar concentrations in treatments with and without fertilizer application declined rapidly and slowly, respectively, after re-irrigation for 7 days (Table 17). This result may be explained that rice plants applied with chitosan and fertilizer can accumulate soluble sugar to adjust osmotic potential in its cells resulting in drought tolerance due to the specific character of chitosan with its ability to elicit a natural defense response and protect various plants from stress conditions (Bhaskara *et al.*, 1999). Moreover, chitosan induces a decrease of K^+ concentration in guard cells resulting in stomatal closure (Bittelli *et al.*, 2001). Chitosan spraying followed by chemical fertilizer application may contribute to drought tolerance by accumulation of soluble sugar.

High soluble sugar concentrations can be maintained beyond the time of drought stress relief (Clifford,1998).

Table 17 Effect of chitosan on soluble sugar accumulation under drought condition. (mg glucose /g leaf dry weight).

Treatment	Early drought period	Severe drought period	Recovery period
Control	0.738	0.698 c	0.589 b
Chitosan application	0.507	0.918 b	0.557 b
fertilizer application	0.672	1.744 a	0.891 a
Chitosan +fertilizer application	0.728	1.783 a	0.898 a
LSD.01	ns	0.192	.201
LSD.05	ns	0.133	.143
C.V.(%)	15.38	4.84	7.41

4. Total nitrogen

Total nitrogen content in rice plants was significantly different between treatments applied and not applied with fertilizer under early drought condition. The nitrogen concentrations in treatment applied with chitosan and fertilizer were greater than those of the others. In the severe drought period, the total nitrogen content in all treatments was generally reduced, particularly, the reduction rates between treatments applied and not applied with fertilizer were still significantly different. In the recovery period, the total nitrogen content in all treatments was slightly increased when irrigation was applied for 7 days after severe drought period. The increasing rates in treatment applied with chitosan and fertilizer were higher than those of the others (Table 18). From this result it can be concluded that the treatment with chitosan and fertilizer application showed the highest total nitrogen content over the others because there were many nitro – compounds in molecules of chitosan and fertilizer resulted in increasing nitrogen content in rice plants. When they were in drought stress condition,

some physiological processes associated with nitrogen accumulation in plants did not function. Hence, the total nitrogen content decreased. The physiological processes started to continue their function again after re-irrigation, thus the total nitrogen levels were gradually increased. This finding was similar to the report of Hayes (1985) who indicated that drought stress may reduce the total nitrogen content of big bluestem and also reported that the leaf total nitrogen concentration peaked in May (rainy Season) (1.64 %), declined rapidly until July (0.60 %) and decreased slowly throughout the summer and fall (water deficit).

Table 18 Effect of chitosan on total nitrogen content under drought condition (%)

Treatment	Early drought period	Severe drought period	Recovery period
Control	1.42 b	0.003 c	0.16 b
Chitosan application	1.69 b	0.004 bc	0.24 b
fertilizer application	2.31 a	0.005 ab	0.33 ab
Chitosan +fertilizer application	2.67 a	0.006 a	0.51 a
LSD.01	0.568	ns	ns
LSD.05	0.390	0.0019	0.188
C.V.(%)	10.26	19.10	32.13

GENERAL DISCUSSION

Chitosan is a natural biopolymer derived from deacetylation (DA) of chitin, a major component of shells of crustacea such as crab, shrimp and crawfish. Chitosan is a nontoxic, biodegradable and biocompatible polymer (Knorr, 1984). Chitosan has wide scope of application in various plants. It not only promotes plant growth and yield but also regulates the immune system of plants (Doares *et al.*, 1995).

In this study, there were four experiments; three pot experiments were conducted in a greenhouse and the another one was carried out in the laboratory. The first experiment studied the effects of molecular weight of chitosan and application method on growth and yield potential of rice cultivar Suphanburi 1. The results of experiment # 1 demonstrated that polymeric chitosan was the most effective type to stimulate growth and increase yield when it was compared with the other chitosan types. It may be explained that the chain of polymeric chitosan was very long. It comprised many glucosamine units ($\approx 100,000 - 1,000,000$ units), which was an unavailable form to be absorbed by rice plants. Therefore, when it was sprayed to rice plants, it needed long periods to be degraded to become an available form (one unit of glucosamine unit), thus the time periods of availability of polymeric chitosan in rice plants were longer than those of the other chitosan types. The benefit of this case was to reduce nutrient losses, because if chitosan was applied in available form, perhaps rice plants could not absorb it in time resulting in losing of excess nutrient. On the other hand, it may be possible that polymeric chitosan containing many nitrogen compounds contributed to add nitrogen which are important in stimulating plant growth. Moreover, the additive effects of chitosan and chemical fertilizer may be considered because chitosan acts as a positive ionic charge, which gives it ability to chemically bind with a negative ionic charge resulting in a slow release action (Sanford, 1992). For the application method, It could be demonstrated that chitosan application by seed soaking followed by foliar spray showed the best effects on growth and rice yield. Rice seed soaked with chitosan may be protected from pathogens and insect infection in soil due to the specific character of chitosan in triggering defense mechanism in plants (Ryan, 1987). Therefore, rice seedlings obtained from treatment with seed soaking followed by foliar spray showed the highest vigor and good plant establishment which was associated with growth and yield potential. A similar result was found in the work of Xue Yan *et al.* (2002) who reported that rapeseed (*Brassica chinensis*) cultivar Aikanggin coated with chitosan showed positive effects on germination index, growth of seedlings and root length. Moreover, seeds of mulberry cultivar Sha2 coated with chitosan solution 3 % increased respiration rate of germinated seed, root vigor, chlorophyll content and peroxidase in seedlings (Xianling *et al.*, 2002).

In the second experiment, the effective type of chitosan (polymeric chitosan) from the first experiment was used to determine the optimum concentration and application frequency. The results showed that chitosan at the rate of 20 ppm applied four times on rice leaves throughout cropping season showed positive effects on growth and rice yield over those of the other concentrations. It may be due to

chitosan is rather specific concentration when applied to each plant species. Some plant species responded with high concentration such as reported by Lee *et al.* (1999) who found that the optimum chitosan concentration for soybean cultivar Junjory was 1,000 ppm. Because the physiological mechanism responses to chitosan in each plant species was different; the optimum concentration for one plant species may not be suitable for another plant species (Yue *et al.*, 2001).

In the third experiment, chitosan concentration at 20 ppm and four foliar applications (the most effective result from the second experiment) were applied to rice plants to determine the appropriate timing for chitosan spraying to trigger defense mechanism of rice plants under drought condition. The results indicated that growth and yield as well as drought recovery of rice plants obtained from treatment sprayed with chitosan before drought showed the most positive effects on rice yield when compared with those of the other treatments including the control. It could be explained that in this treatment, all physiological mechanism processes in rice plants were still functional to some degree because drought condition was not occurred while chitosan was sprayed to rice plants. Therefore, it could be absorbed and changed into available form by some enzymes resulted in triggering the defense mechanism by stomata closing or phytoalexin production (Bittelli *et al.*, 2001; Walker – Simmons *et al.*, 1983). The damage of rice plants from drought in this treatment was less than the control and the others sprayed with chitosan during and after drought. In the control and the other treatments they showed a negative effect on drought recovery and yield. It could be demonstrated that rice leaves in these treatments were tightly rolled and some physiological processes were interrupted due to water deficit. There was only some portion of chitosan absorbed by rice plants, therefore it was not enough to induce defense mechanism responses which are associated with growth and yield decreasing.

The fourth experiment aimed at detecting some physiological responses of rice plants after chitosan application under various drought levels. The results revealed that the application of chitosan and fertilizer showed positive effects to protect rice plants from drought by accumulation of some solutes such as proline and soluble sugar over the other treatments. It could be explained that the additive effects of chitosan and fertilizer may enable rice plants to tolerate to drought better than the other treatments. The plant species generally treated with chitosan are stimulated to produce some secondary metabolites involved in plant defense responses (Loschke *et al.*, 1983; Walker – Simmons *et al.*, 1983). Therefore, rice plants sprayed with chitosan could adjust some mechanism in their cells to survive by accumulation of proline and soluble sugar which were associated with osmotic adjustment in plant cells resulting in the maintenance of cell water potential levels under drought condition. The activity of nitrate reductase of treatment sprayed with chitosan and fertilizer was less than those of the others under severe drought because the growth of rice plants in this treatment was rapid, so that when it faced severe drought; it became seriously wilted which resulted in photosynthesis reduction. The nitrate reductase activity was reduced because it required NADPH or NADH, product of photosynthesis, as an electron donor (Evans and Nelson, 1953). Furthermore, transpiration rate was decreased due to leaf rolling resulting in reduction of nitrate

absorption which associated with the activity of nitrate reductase. The total nitrogen content in treatment sprayed with chitosan and fertilizer was higher than those of the others due to the additive effects of chitosan and fertilizer. There are many nitrogen compounds in chitosan molecules. It may be possible that both chitosan and fertilizer contributed to increase total nitrogen content in rice plants.

From this study, there are recommendations for chitosan application that

1. Application of chitosan in plants for increasing yield should soak the seed before planting and then followed by foliar spraying because it shows significant differences in yield increasing over the control. It is easy for practices and not expensive for investment.
2. Application of chitosan in plants for severe damage reduction from drought should spray before drought appearance. Because drought appearance can happen all the times and out of control. Therefore, the plants treated with chitosan are safe or less damage when they are in drought condition. Furthermore, drought recovery in plants applied with chitosan is faster than control.
3. This study was conducted with pot experiment, so that some factors may be controlled easier than in field experiment which associated with the results. To confirm the results, the experiment should be carried out in farmer field condition for getting exact results.

CONCLUSION

1. Application of polymeric chitosan by seed soaking before planting and four foliar applications at seedling, tillering, panicle initiation and heading stage showed positive effects on growth grain yield and yield components of rice cultivar Suphanburi 1

2. Application of polymeric chitosan at 20 ppm with four foliar applications throughout cropping season showed the highest growth, yield and yield components.

3. Application of polymeric chitosan by foliar spraying before drought period in each growth stage may reduce the severe damage from drought and allowed drought recovery better than those of treatments applied with chitosan during and after drought.

4. In early drought period, chitosan did not affect nitrate reductase activity, proline accumulation and soluble sugar accumulation whereas total nitrogen content was affected by chitosan application.

5. In severe drought period, proline and soluble sugar accumulation were increased but nitrate reductase activity was decreased particularly in treatment applied with chitosan and fertilizer. Total nitrogen content was decreased in all treatments but the highest was observed from treatment with chitosan and fertilizer application.

6. For the recovery period, the proline and soluble sugar accumulation were rapidly decreased in treatment applied with chitosan and fertilizer whereas nitrate reductase activity and total nitrogen content gradually increased in all treatments but the highest was observed in treatment applied with chitosan and fertilizer.

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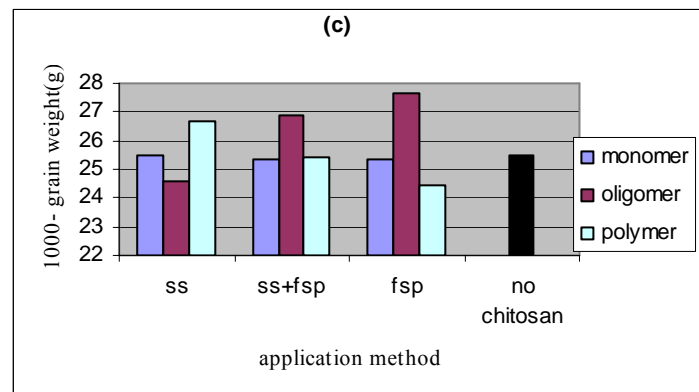
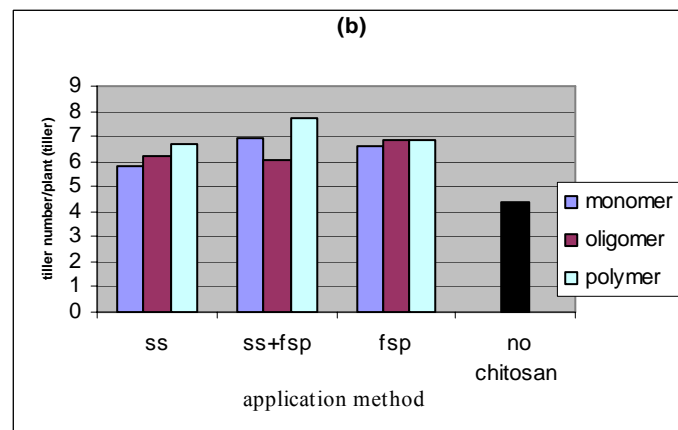
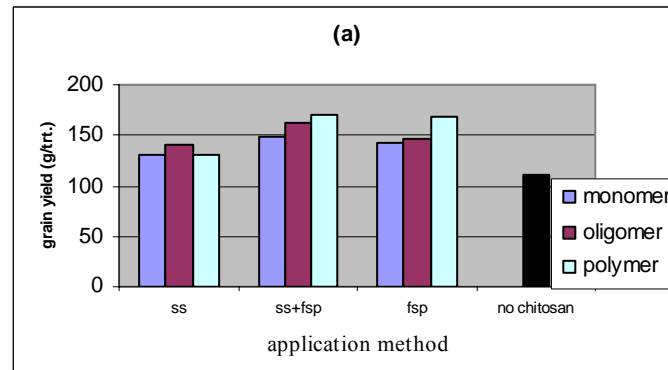
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APPENDIXES

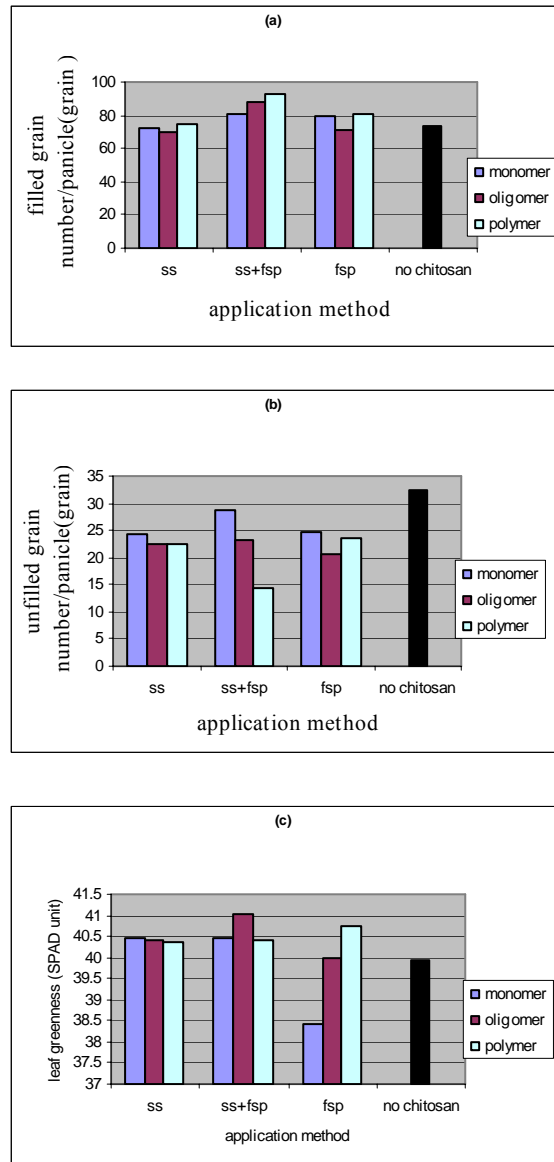


Appendix Figure 1 Effects of chitosan with different molecular weights and application methods on rice yield (a), tiller number (b) and 1,000- grain weight (c).

ss = seed soaking

ss + fsp = seed soaking + foliar spray

fsp = foliar spray

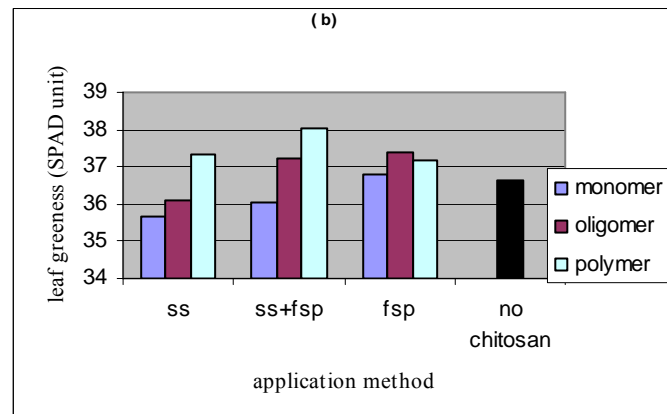
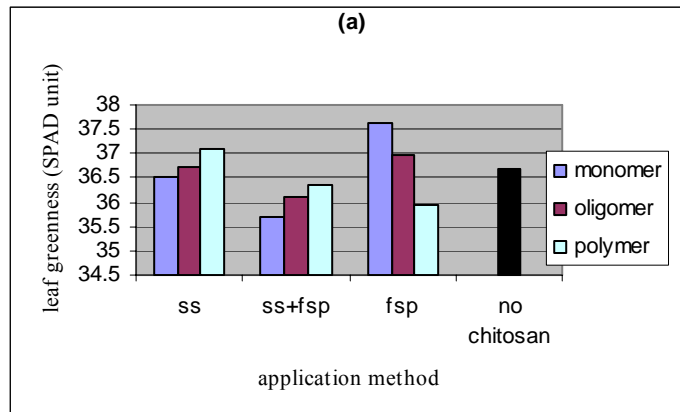


Appendix Figure 2 Effects of chitosan with different molecular weights and application methods on filled grain (a), unfilled grain (b) and leaf greenness at seedling stage (c).

ss = seed soaking

ss +fsp = seed soaking+foliar spray

fsp = foliar spray

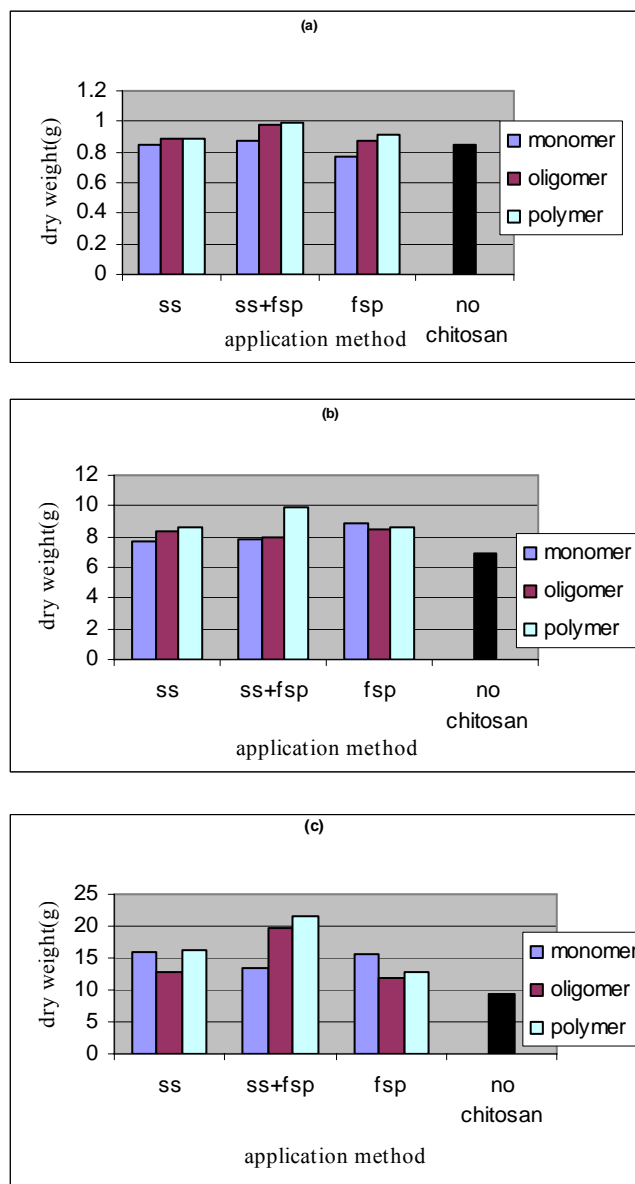


Appendix Figure 3 Effects of chitosan with different molecular weights and application methods on leaf greenness at tillering stage (a) and panicle initiation stage (b).

ss = seed soaking

ss +fsp = seed soaking+foliar spray

fsp = foliar spray

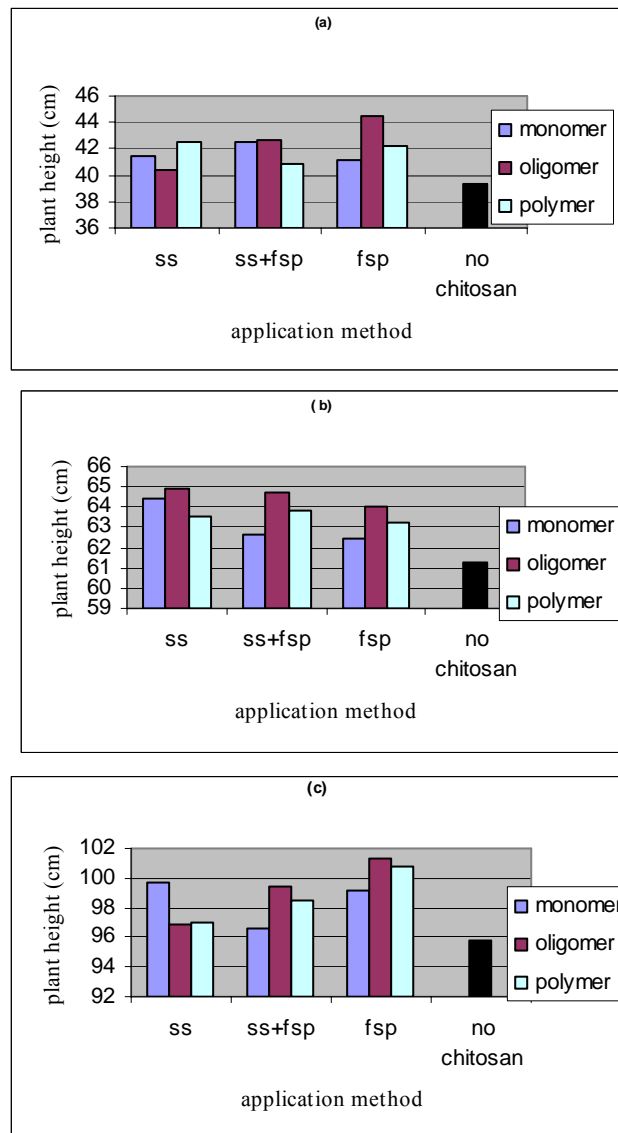


Appendix Figure 4 Effects of chitosan with different molecular weights and application methods on dry weight at seedling stage (a), tillering stage (b) and panicle initiation stage (c).

ss = seed soaking

ss +fsp = seed soaking+foliar spray

fsp = foliar spray

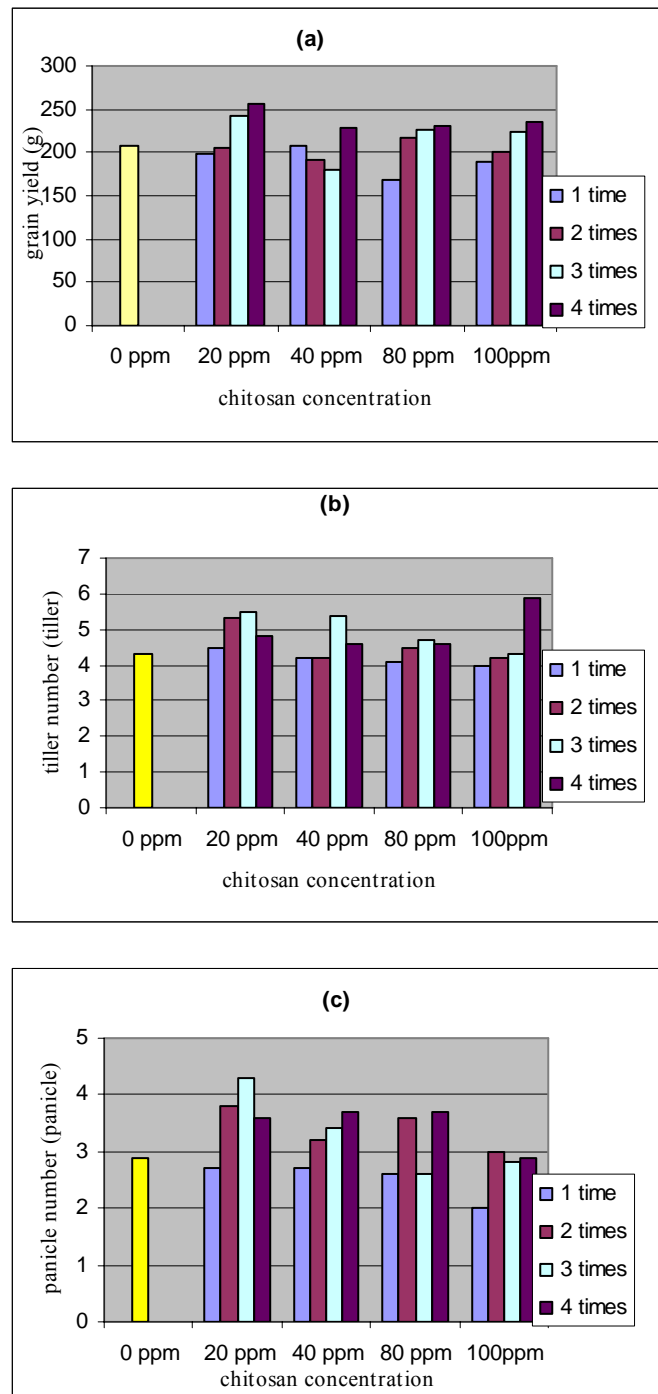


Appendix Figure 5 Effects of chitosan with different molecular weights and application methods on plant height at seedling stage (a), tillering stage (b) and harvesting stage (c).

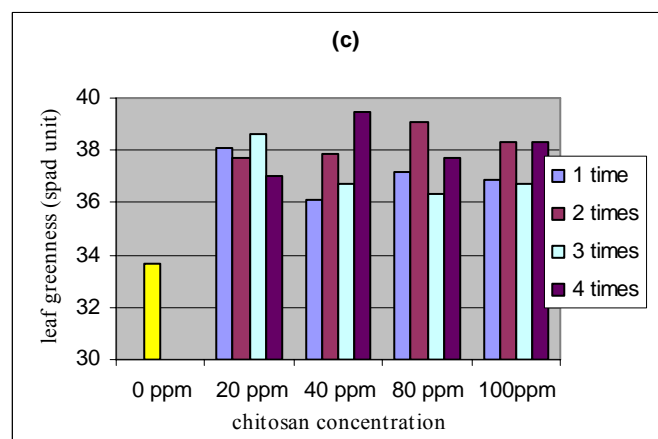
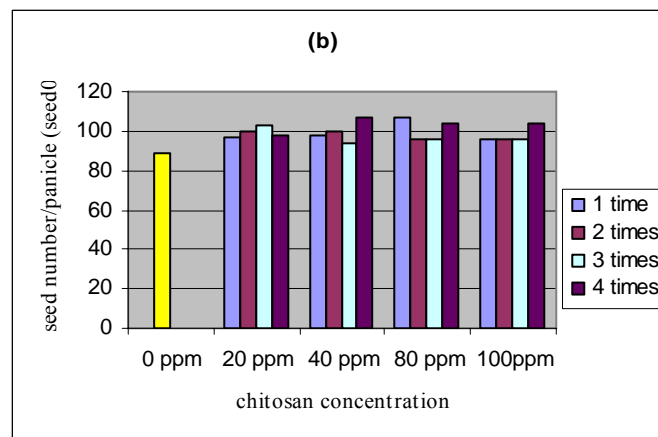
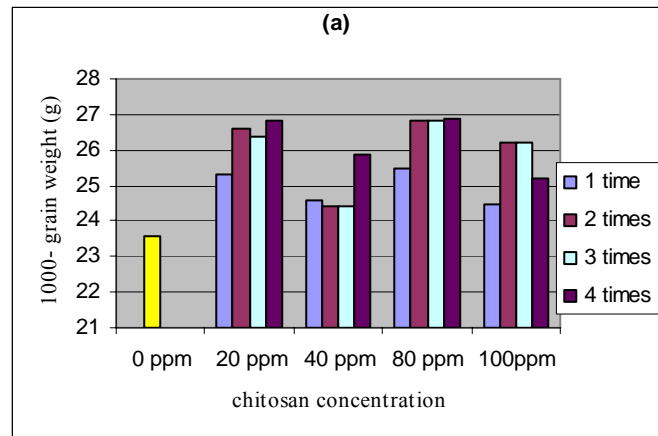
ss = seed soaking

ss +fsp = seed soaking+foliar spray

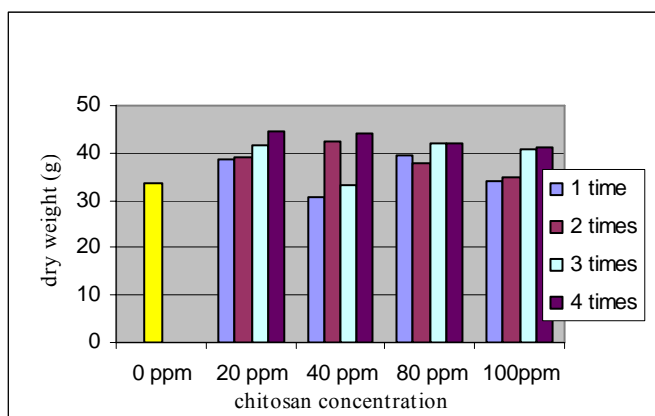
fsp = foliar spray



Appendix Figure 6 Effects of concentrations and application frequencies on rice yield (a), tiller number (b) and panicle number (c).



Appendix Figure 7 Effects of concentrations and application frequencies on 1,000- grain weight (a), seed number (b) and leaf greenness (c)



Appendix Figure 8 Effects of concentrations and application frequencies on dry matter accumulation.

Chitosan concentrations (factor A)

20 ppm

40 ppm

80 ppm

100 ppm

Foliar application frequencies (factor B)

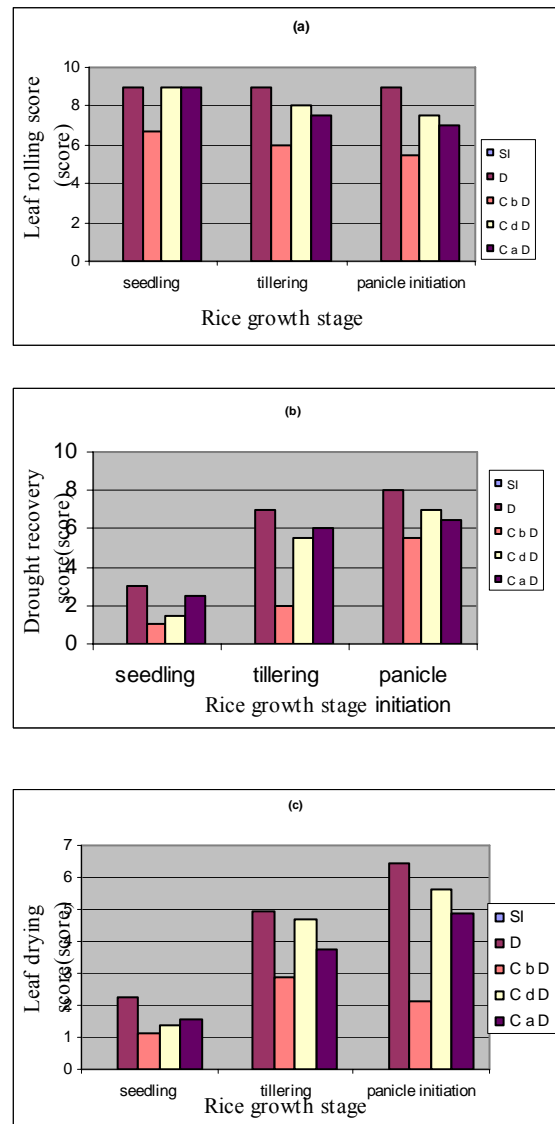
1 time = at seedling stage

2 times = at seedling and tillering stages

3 times = at seedling, tillering and panicle initiation stages

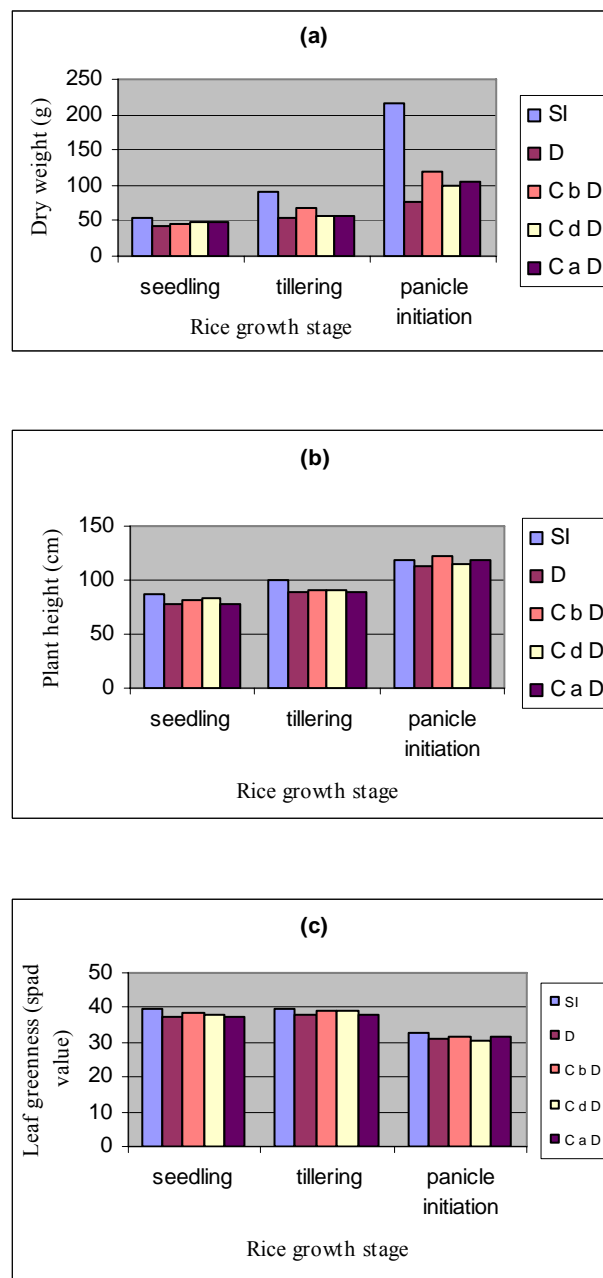
4 times = at seedling, tillering, panicle initiation and heading stages

Control = no chitosan application



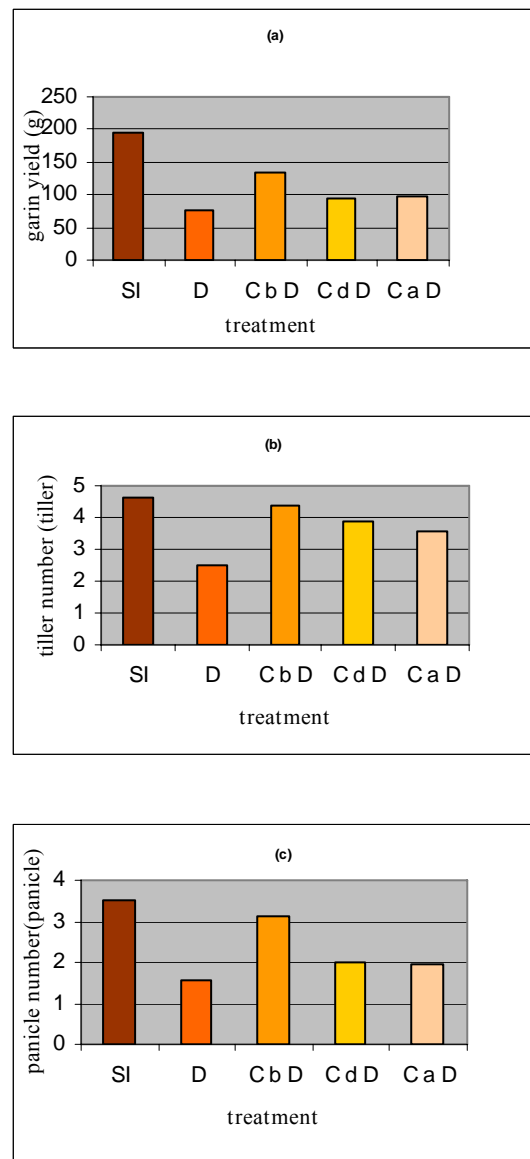
Appendix Figure 9 Effect of chitosan on leaf rolling (a), drought recovery (b) and leaf drying (c) of rice under drought condition.

- SI = sufficient irrigation
- D = drought without chitosan
- CbD = chitosan was applied before drought
- CdD = chitosan was applied during drought
- CaD = chitosan was applied after drought



Appendix Figure 10 Effect of chitosan on dry weight (a), plant height (b) and leaf greenness (c) of rice under drought condition.

- SI = sufficient irrigation
- D = drought without chitosan
- CbD = chitosan was applied before drought
- CdD = chitosan was applied during drought
- CaD = chitosan was applied after drought



Appendix Figure 11 Effect of chitosan on grain yield (a), tiller number (b) and panicle numbers of rice under drought condition.

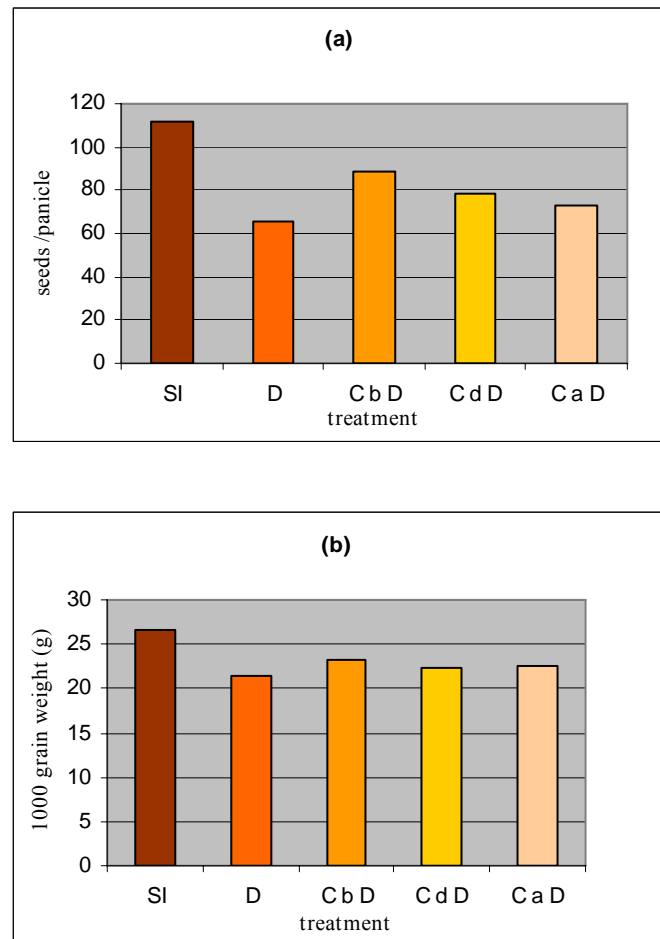
SI = sufficient irrigation

D = drought without chitosan

CbD = chitosan was applied before drought

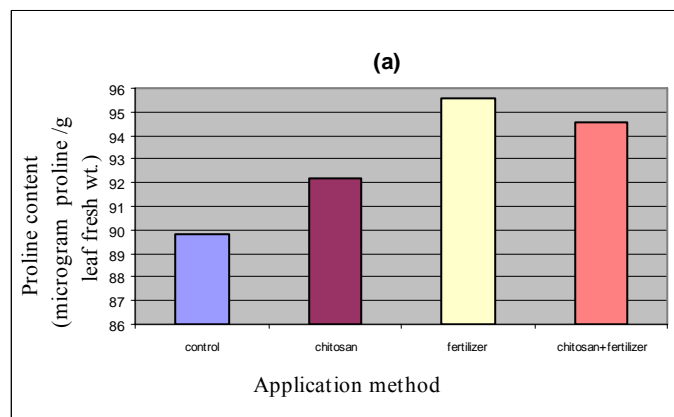
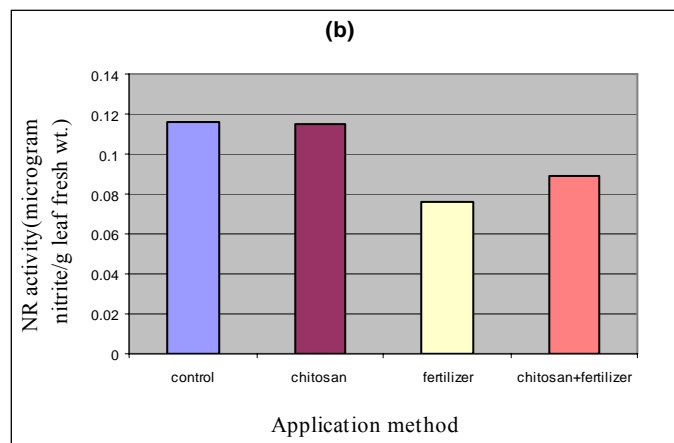
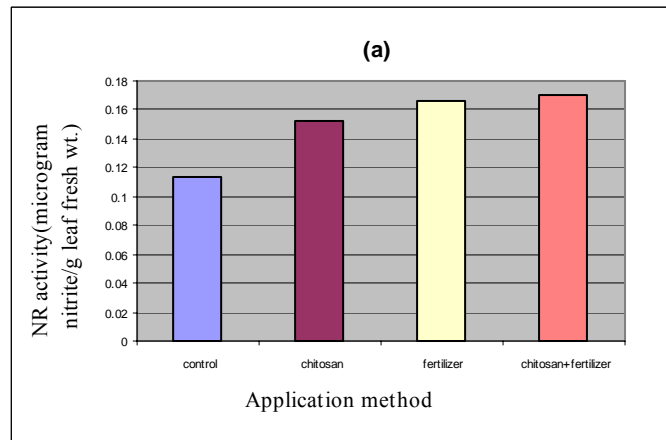
CdD = chitosan was applied during drought

CaD = chitosan was applied after drought

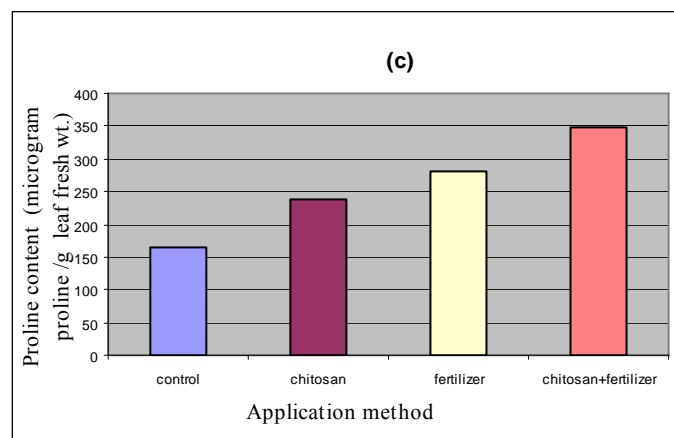
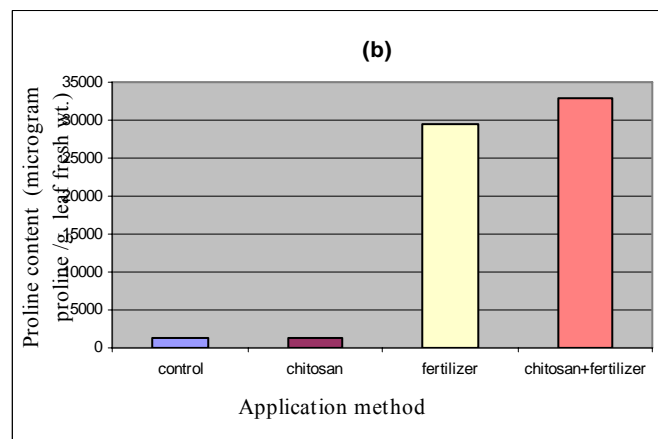
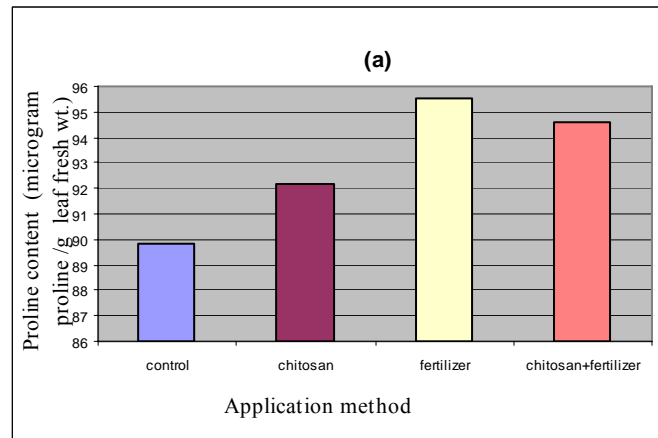


Appendix Figure 12 Effect of chitosan on seed numbers (a) and panicle numbers (b) of rice under drought condition.

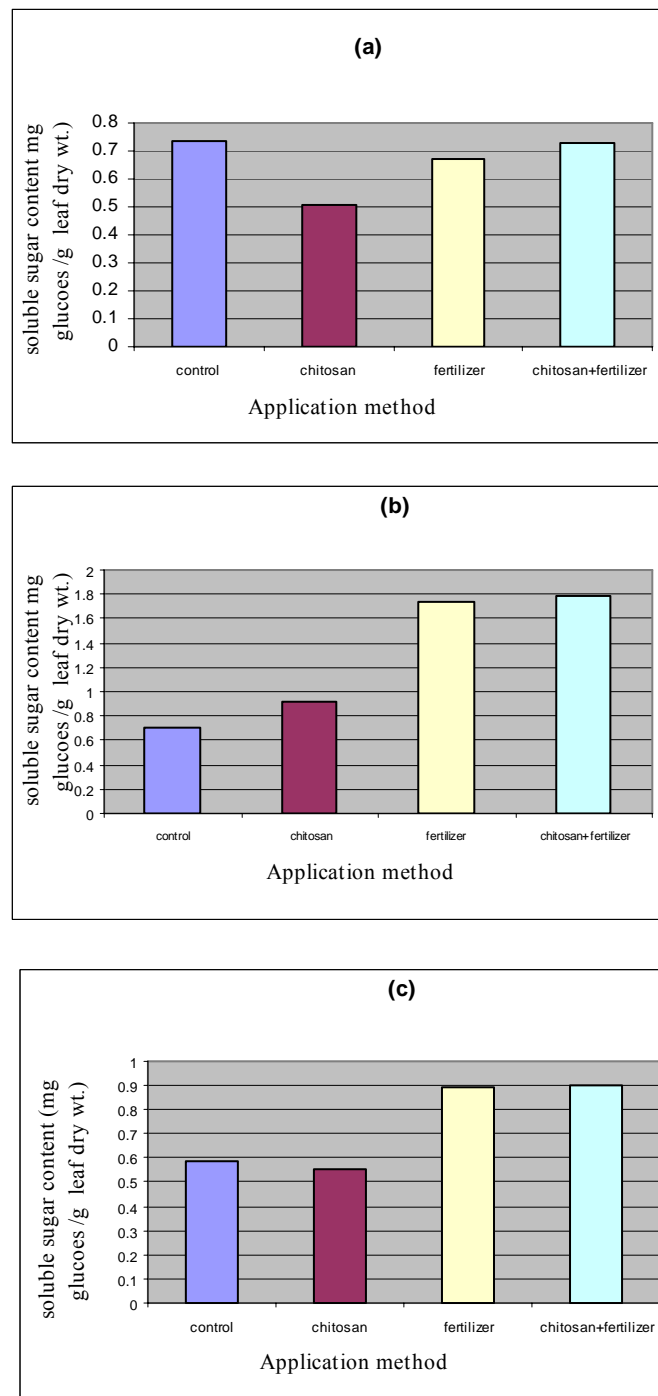
- SI = sufficient irrigation
- D = drought without chitosan
- CbD = chitosan was applied before drought
- CdD = chitosan was applied during drought
- CaD = chitosan was applied after drought



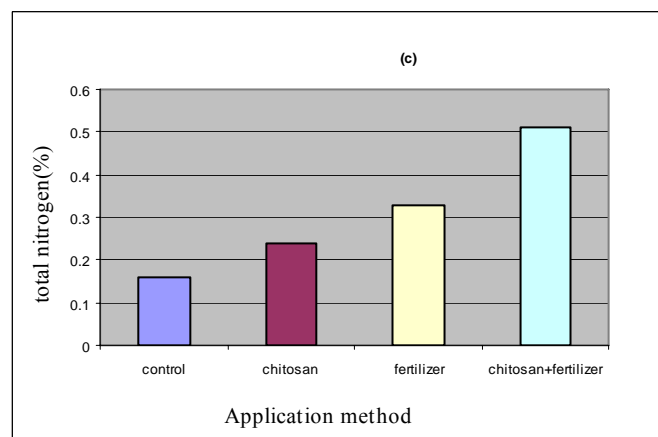
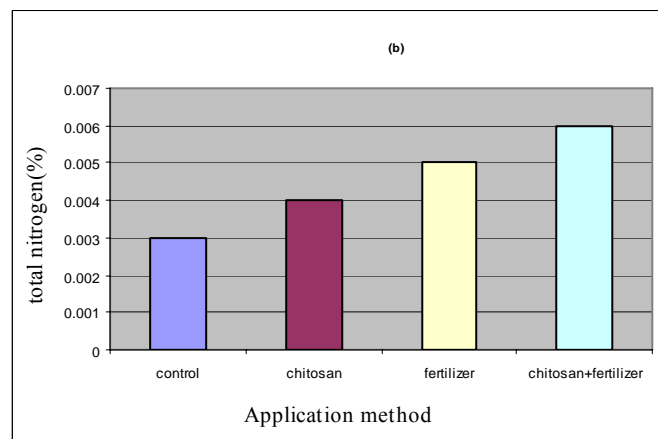
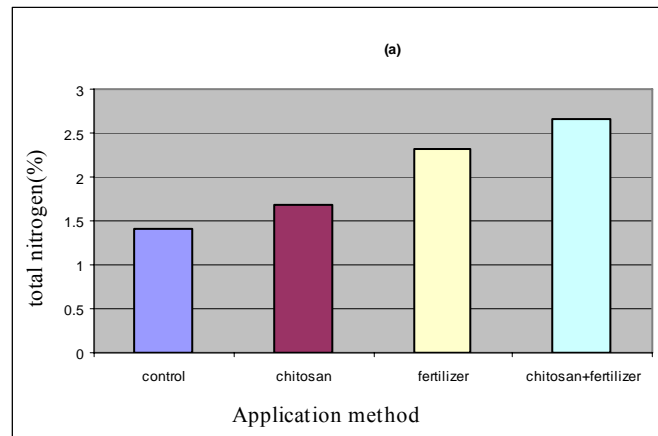
Appendix Figure 13 Effect of chitosan on nitrate reductase activities under early drought (a), severe drought and recovery period (c).



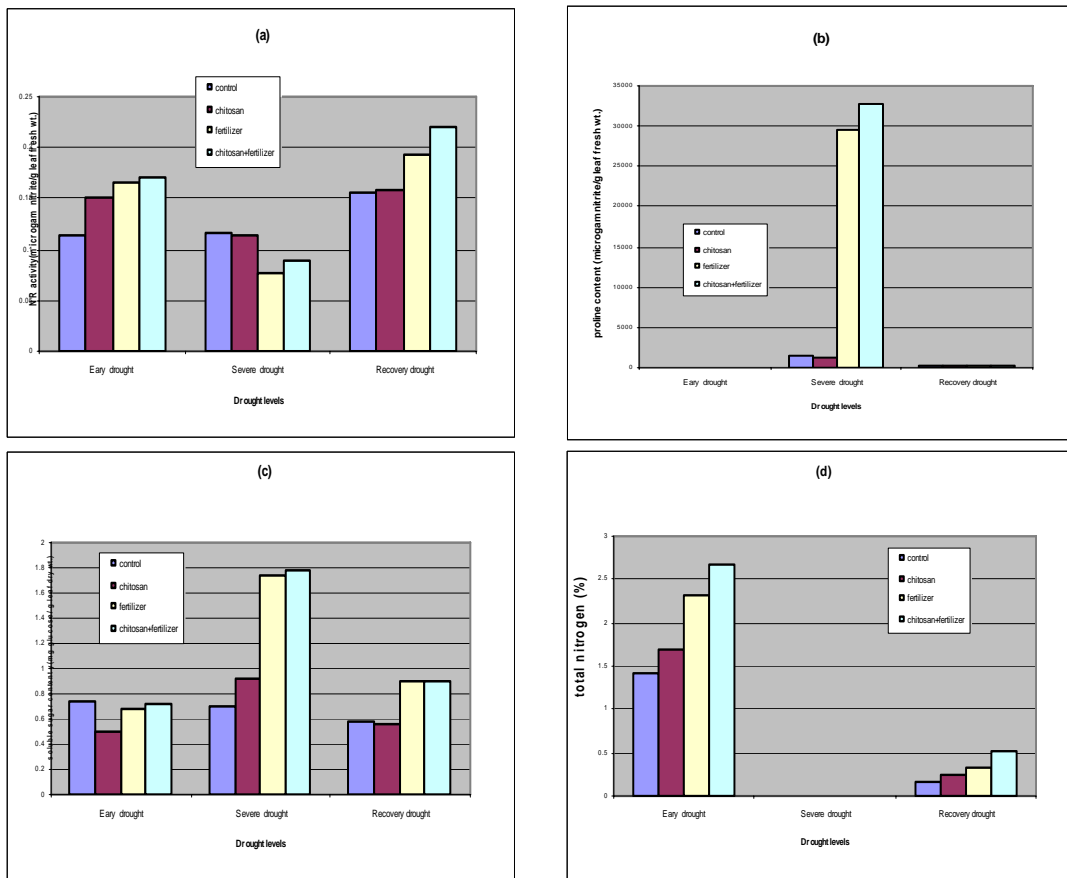
Appendix Figure 14 Effect of chitosan on proline accumulation under early drought (a), severe drought (b) and recovery period (c).



Appendix Figure 15 Effect of chitosan on soluble sugar accumulation under early drought (a), severe drought (b) and recovery period (c).



Appendix Figure 16 Effect of chitosan on total nitrogen content under early drought (a), severe drought (b) and recovery period (c).



Appendix Figure 17 Effect of chitosan on nitrate reductase (a), proline (b), soluble sugar (c) and total nitrogen (d) under various drought levels.