SHORT COMMUNICATION

CHITOSAN OLIGOMERS AS BIO-STIMULANTS TO ZUCCHINI (*CUCURBITA PEPO*) SEEDS GERMINATION

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Germination studies of untreated and chitosan-coated zucchini seeds are presented. Two samples of chitosan, denoted as C2 (Mw = 34 kDa) and C5 (Mw = 28 kDa), were prepared by a potassium periodate-depolymerization process applied to a commercial sample, denoted as C0 (Mw = 300 kDa). The effect of these chitosan samples on the germination of zucchini seeds was investigated (a) immediately after they were removed from the fruit and (b) during a 6-month storage period. Results clearly showed that zucchini seeds coated with two layers of C5 sample presented both lower average germination time and higher accumulated germination percentage at day 2 of the germination test. Additionally, optimal values for storage time and concentration of C5 coating solution were obtained by using a central composite rotatable design. Values found were as follows: (a) 2.90 months and 0.76% (w/v) for seeds stored at room temperature; and (b) 4.47 months and 0.65% (w/v) for seeds stored under refrigeration. Thus, the germination stimulant property of these chitosan oligomers has been demonstrated for this particular system, and, more comprehensively, a valuable experimental result was achieved to support the recent proposal to include these materials into the oligosaccharins group, a new type of plant growth promoter.

Key words: zucchini seed, germination stimulant, chitosan seed coating, plant growth promoters, average germination time

Biotechnological and scientific research related to bio-agrochemicals that fulfills its role in an environmentally friendly manner is a field of intense activity. Chitosan (Figure 1), a polysaccharide found naturally in the cell walls of some fungi, is among the most promising biomaterials for these purposes (Lárez-Velasquez 2008; El Hadrami *et al*. 2010). It can be considered a copolymer consisting of glucosamine and N-acetyl-glucosamine repetitive units (fraction of glucosamine units = deacetylation degree = DD > 0.50) linked randomly through β-1,4 bonds.

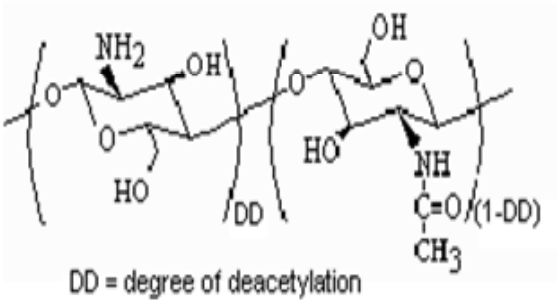

Figure 1. Chitosan structure

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Chitosan has been studied in recent years as a natural agrochemical for applications in areas such as seed coating (Freepons 1997), preservation of fruits and vegetables (Assis & Pessoa 2004), fungicide (Allan & Hadwiger 1979), growth bio-stimulant (Burrows *et al*. 2007), elicitor (Franco & Iriti 2007), inducer of resistance to pathogens (Ozeretskovskaya *et al*. 2002), etc. Chitosan effectiveness as germination stimulant has been also proved for a variety of plant species such as cotton, maize (Tingda *et al*. 1994), wheat (Bhaskara *et al*. 1999), cucumber, chili, pumpkin, cabbage (Chandrkrachang 2002), okra (Jaybhay *et al*. 2010), artichoke (Ziani *et al*. 2010), etc.

In this paper, studies of chitosan-coated zucchini seeds were carried out with the aim of investigating the effect of molecular weight and concentration of chitosan on zucchini seeds germination. Zucchini seeds were previously coated with two layers of different chitosan samples and dried and stored during 6 months under two storage conditions: ambient and refrigerator. The effect of molecular weight on average germination time (t_g) was established for seeds in both storage conditions. Optimal values for storage time (t_{st}) and chitosan concentration [C] were also found by using a central composite rotatable design (CCRD).

Materials

Chemicals: Chitosan (Fluka BioChemika, M_r ~ 150.000) was employed as starting material to obtain two additional depolymerized samples by using KIO_4 (Merck pro analysis, 99.8%) as a depolymerizing agent; acetic acid (Riedel de Haen, 99.8%), sodium acetate (pro analysis, 99%), isopropyl alcohol (Fisher Scientific), sodium hydroxyde (EKA Chemicals, 99%), sodium hypochlorite (commercial antiseptic solution, 5%) were used as received from suppliers.

Seeds: They were obtained from harvested fruits of the second generation of certified seeds (Vilmorin, France) purchased in a local farm store. These were grown and harvested (after 4 months), without agrochemicals treatment, in a farm located at El Paramillo, Merida State, Venezuela. For sterilization, manually extracted seeds were treated as follows: (a) removal of the surface membrane, (b) immersion in a 2% NaClO solution with manual stirring during 20 minutes, (c) removal of NaClO by five consecutive washings in sterile distilled water, and (d) drying at room temperature for 30 minutes in a laminar flow hood. Untreated and chitosan-coated zucchini seeds were kept until use in closed paper bags under two storage conditions: ambient (~ 20°C) and refrigerator (~ 4°C).

Chitosan characterization

Molecular weight determination: (a) viscosity average molecular weight for starting chitosan (C0, M_v~ 195 kDa) was determined by obtaining its intrinsic viscosity [η] in CH_3COOH(0.30M)/CH_3COONa(0.20M) aqueous solution at 25°C (Cannon 150-D503 capillary viscometer and Gallenkamp thermostated bath) and using $K = 7.4 \times 10^{-4}$ and $\alpha = 0.76$ values in the Mark-Houwink-Sakurada equation (Rinaudo *et al*. 1993); (b) average molecular weight was determined by size exclusion chromatography (SEC), employing a HPLC Waters 600E chromatograph fitted with a Waters 410 differential refractometer and Waters Ultrahydrogel (250 and 2000) columns, employing the aqueous solvent used for the viscosimetric study as eluent. A calibration curve was obtained employing standard samples of poly(ethylene oxide).

Deacetylation Degree (DD) determination: (a) a conductimetric titration method (Hernández *et al*. 2009) using standardized NaOH was employed to quantify DD in the starting chitosan sample (DD = 0.771), and (b) two FTIR approaches were employed for DD determination (Brugnerotto *et al*. 2001; Baxter *et al*. 1992) obtaining an average value for DD = 0.787.

Chitosan depolymerization

Reactions were performed through a slight modification of the procedure reported by Lárez-Velásquez & Zambrano-Díaz (2011) which can be summarized as follows: 2.5 g of C0 were dissolved during 12 hours in a 250 mL volumetric flask with water and 1 mL of acetic acid; the resulting solution was then vacuum-filtered through a fritted-glass funnel (Sovirel 2) and treated with the necessary stoichiometric amount of KIO_4 to achieve a predetermined oxidation percentage, that is, 2% and 5% of amine group equivalents, obtaining two final chitosan samples (C2 and C5, respectively). C2 and C5 molecular weight decreasing was confirmed by SEC, injecting

the depolymerized final solutions immediately after they have completed their KIO_4 treatment time. SEC conditions were similar to those for characterization of the starting chitosan.

Seed coating procedure

A previous germination study was conducted in order to optimize the number of chitosan layers to be applied to seeds by using one, two, and three layers of C0. In this case, all layers were deposited from a C0 solution of concentration 0.55% (w/v) in aqueous acetic acid 0.42% (w/v). A typical procedure for seed coating can be summarized as follows: dry seeds were arranged on a plastic grid, and a first coating was applied on the exposed side of the seeds using a sprayer containing the adequate chitosan solution; after 20 minutes of air drying seeds were turned over and sprayed. The treatment was repeated to get seeds coated with two layers of chitosan.

Germination studies

All germination experiments were performed by triplicate, placing five seeds in a Petri dish containing the germination substrate (cotton and adsorbent paper) previously sterilized by autoclaving (120°C, 15 pound pressure, 15 minutes). After addition of 20 mL of sterilized water, Petri dishes were sealed with parafilm (Pechiney PM-996) and placed under aseptic conditions in a laminar flow hood. Verification of seed germination was carried out each 24 hours in order to obtain values of t_g and accumulated germination percentage at day 2 (AG%). Germination was considered positive when radicle emergence was observed. Selected values for t_{st} and [C] during this study are presented in Table 2. They were chosen by application of a CCRD that considered a range from 1 to 6 months for t_{st} and from 0.1 to 1% (w/v) for [C].

Figure 2 shows SEC traces for starting C0 solution and the resulting chitosan solutions after KIO_4 treatments (C2 and C5). As it can be clearly observed, C2 and C5 traces show a significant shift towards lower molecular weight values, the largest displacement corresponding to C5, in accordance with the higher amount of KIO_4 added to this one. Computed M_w values for C0, C2, and C5 were around 229 kDa, 34 kDa, and 28 kDa, respectively. Although C2 and C5 M_w values are close, their SEC traces clearly indicate that C5 contains a larger fraction of oligomers.

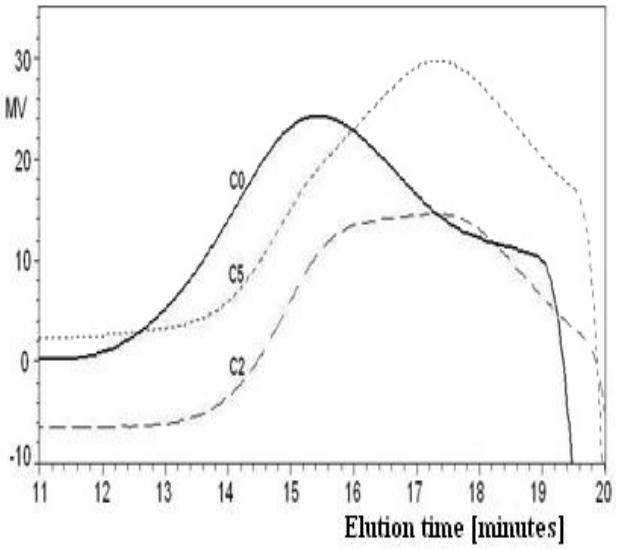

Figure 2. SEC traces for C0 (without KIO_4 treatment) and C2 and C5 solutions obtained after KIO_4 treatment

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Results from optimization study of number of chitosan layers on zucchini seeds

Number of chitosan layers	AG% (day 2)*	SE
0 (control)	33.3	5.44
1	40.0	3.14
2	57.8	2.83
3	37.8	4.34

SE – standard error = $SD/n^{1/2}$ where SD = standard deviation and n = number of experiments

* – accumulated germination percentage at day 2

The previous germination study (Table 1) demonstrated that two-layered chitosan coatings generate beneficial effects on germination of zucchini seeds rather than either untreated or single- or three-layered chitosan coated seeds. Therefore, the complete work was carried out employing seeds coated with two layers. Table 2 shows results obtained for average t_g using uncoated and chitosan-coated zucchini seeds stored at room temperature. t_{st} and [C] values emerge from levels of the CCRD applied; t_g values are computed applying the next equation:

$t_g = (n_1d_1 + n_2d_2 + n_3d_3 + n_4d_4) / (n_1 + n_2 + n_3 + n_4) = \Sigma n_i d_i / \Sigma n_i$

where: n_i – number of germinated seeds on i day (d_i),
d_i – values in the above sum will range between 1 and 4 since seeds exhibited a maximum t_g – 4 days during the course of the study.

Figure 3 shows the comparative results on t_g for untreated and chitosan-coated zucchini seeds, immediately after seeds were sterilized and dried, that is, for a $t_{st} = 0$. The t_g value for untreated seeds was obtained as an average from experiments with 5 seeds (3 replicates) as it is shown in Table 2; the t_g values for chitosan-coated seeds were obtained as an average for each sample of studied chitosan by using data coming from all assayed concentrations (Table 2). Although differences in t_g values for chitosan-coated seeds were not statistically significant, it can be appreciated that chitosan coating on zucchini seeds leads to shorter t_g, becoming the effect more noticeable for C5.

Similarly, Figure 4 shows comparative results over the complete time study for untreated and chitosan-coated zucchini seeds, under the storage conditions evaluated. Values of t_g for untreated zucchini seeds were obtained as it was previously described for t_{st} values shown in Table 2; values of t_g for chitosan-coated seeds were obtained as an average for each sample of chitosan studied at each of the t_{st} and [C] values shown in Table 2. In this case, evaluation of untreated (1–6) showed, for both storage conditions – ambient (a) and refrigerator (r), shorter t_g than untreated (0). It could be attributed that zucchini seeds achieve lower values of t_g (higher average vigor = v = 1 / t_g) over a given stor-

T a b l e 2

t_g results obtained during germination studies of untreated and coated zucchini seeds for concerned parameters emerging from application of CCRD. Storage seed condition: room temperature

Treatment chitosan	[C] % [w/v]	t_g [days]*					
		0.0**	1.0** (–1.414)***	1.7** (–1.000)	3.5** (0.000)	5.3** (1.000)	6.0** (1.414)
Untreated	---	2.93 ± 0.23	2.60 ± 0.40	2.80 ± 0.20	2.33 ± 0.12	2.53 ± 0.51	1.87 ± 0.50
C0	0.10 (–1.414)***	2.27 ± 0.23	2.13 ± 0.23		2.47 ± 0.31	2.33 ± 0.12	2.40 ± 0.53
	0.23 (–1.000)	3.20 ± 0.53		2.27 ± 0.12			
	0.55 (0.000)	3.13 ± 0.42			2.27 ± 0.12		
	0.87 (1.000)	2.53 ± 0.42		2.27 ± 0.12		2.07 ± 0.42	
	1.00 (1.414)	2.67 ± 0.31			2.47 ± 0.42		
C2	0.10 (–1.414)	2.73 ± 0.64	2.33 ± 0.31		2.40 ± 0.35	2.53 ± 0.31	2.27 ± 0.31
	0.23 (–1.000)	2.60 ± 0.53		2.40 ± 0.20			
	0.55 (0.000)	3.00 ± 0.35			2.07 ± 0.12		
	0.87 (1.000)	2.80 ± 0.35		2.27 ± 0.12		2.20 ± 0.53	
	1.00 (1.414)	2.67 ± 0.31			2.13 ± 0.50		
C5	0.10 (–1,414)	2.33 ± 0.12	2.07 ± 0.12		2.33 ± 0.12	1.73 ± 0.21	2.00 ± 0.00
	0.23 (–1.000)	2.47 ± 0.58		1.87 ± 0.31			
	0.55 (0.000)	2.07 ± 0.12			2.00 ± 0.00		
	0.87 (1.000)	2.20 ± 0.35		2.67 ± 0.46		1.60 ± 0.40	
	1.00 (1.414)	2.47 ± 0.31			1.53 ± 0.23		

*all reported values were obtained from 3 replicates (5 seeds each)
**storage time = time at which germination assay was conducted (months)
***values between parentheses represent levels emerging from CCRD

age time, as it has been reported for other seeds, for example, *Passiflora mollissima* (Giraldo & Aristizabal 2008).

On the other hand, all the chitosan-coated seeds showed a positive effect on t_g, with values lower than those of untreated seeds evaluated in the same period, although this effect was more noticeable for C5. The t_g values for C5-coated seeds were statistically significant, p = 95 (ambient) and 98 (refrigerator). The greater activity to stimulate germination observed on seeds coated with C5 perfectly agrees with previous reports on the use of chitin oligomers (Hirano 1996) and chitosan (Dzung 2004), which have recently led to include them as plant growth

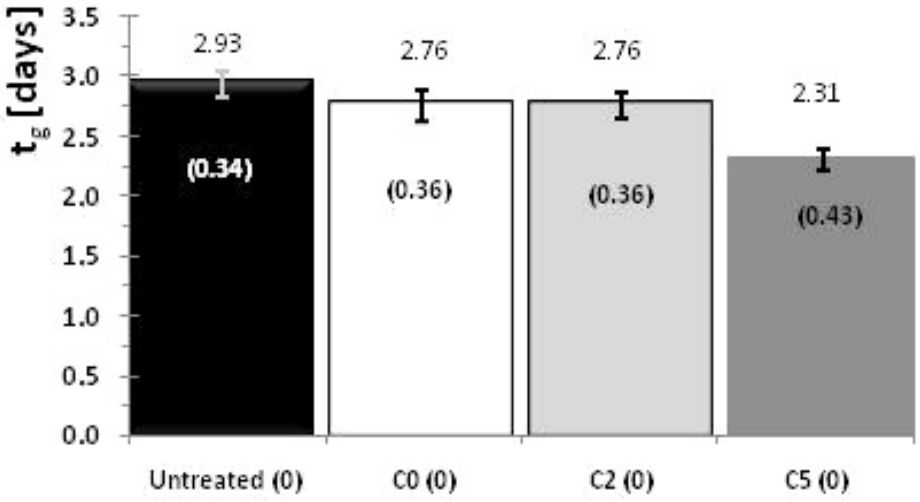

Figure 3. Average t_g for untreated and chitosan-coated zucchini seeds at t_{st} = 0. Values between parentheses represent the average vigor (v) of seeds; bars represent standard error

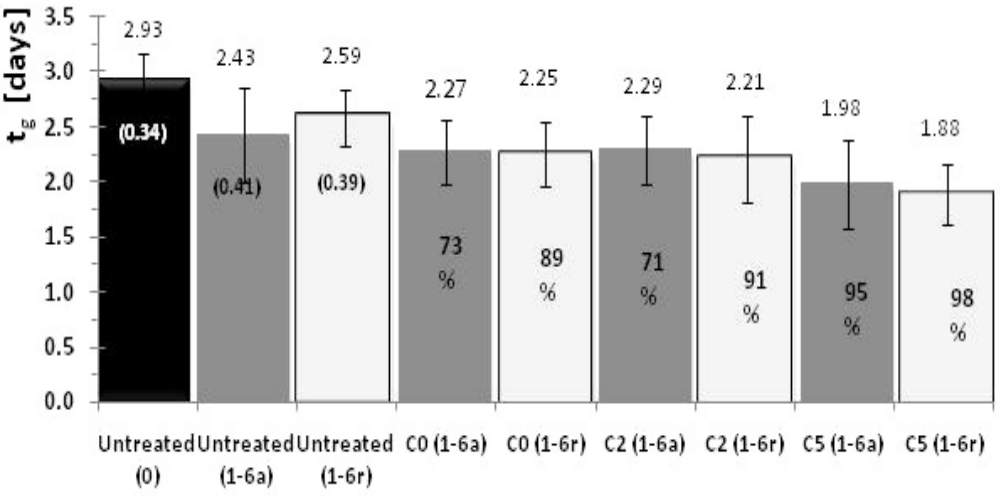

Figure 4. Average t_g for untreated and chitosan-coated zucchini seeds during a t_{st} interval of 1–6 months. Values denoted as (1–6a) and (1–6r) refer to seeds stored at room temperature (ambient) and refrigerator, respectively. Percentage values within the columns indicate the level of confidence in relation to the respective control. Values between parentheses indicate average vigor

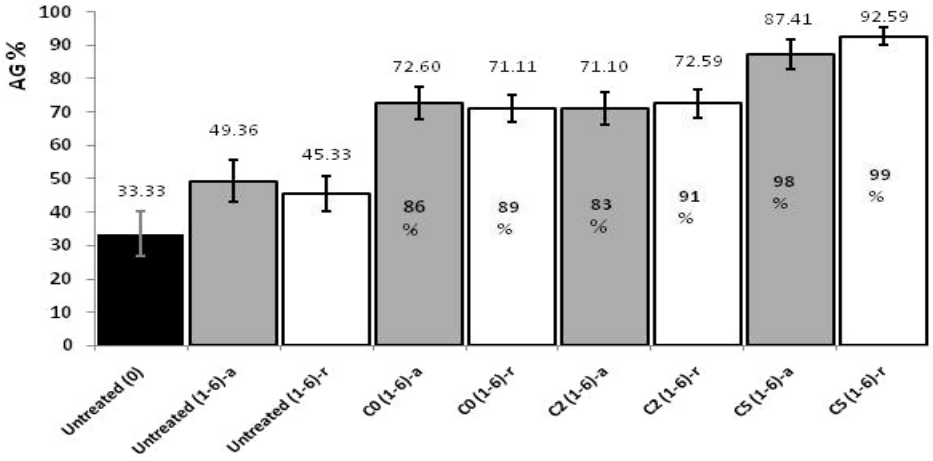

Figure 5. AG% for untreated and chitosan-coated zucchini seeds during a t_{st} interval of 1–6 months. Values denoted as (1–6a) and (1–6r) refer to seeds stored at room temperature (ambient) and refrigerator, respectively. Percentage values within the columns indicate the level of confidence related to the respective control

promoters belonging in the oligo-saccharine group (Dzung 2011).

The above results show that coverage with C5 was the most effective treatment to stimulate germination of studied zucchini seeds. It was also confirmed by AG% studies carried out considering the number of germinated seeds on day 2 (Figure 5). It can be observed that AG% values for both storage conditions of C5-treated seeds, ambient -C5(1–6a)- and refrigerator -C5(1–6r)- were highest and also showed greater statistical significance with respect to untreated seeds.

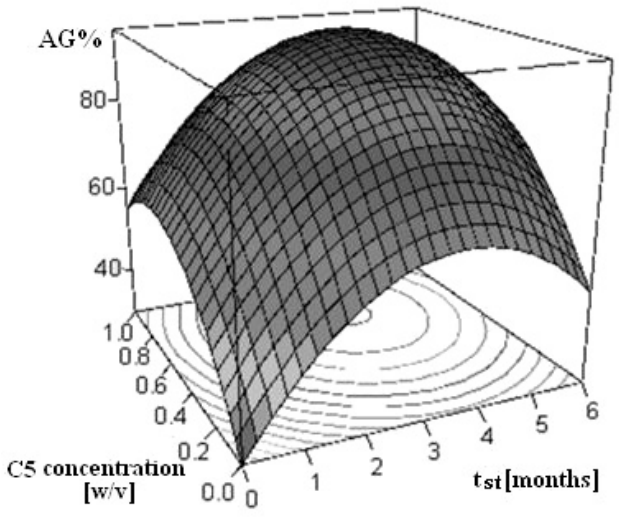

Figure 6. AG% surface response for C5-coated zucchini seeds stored at refrigerator (~4°C)

Accordingly, a more detailed AG% data analysis for this system along the storage time was also carried out via CCRD. Figure 6 shows the surface response obtained from the refrigerated seeds; optimal values obtained from this for t_{st} and C5 concentration were 4.47 months and 0.65% (w/v), respectively. A similar analysis for seeds stored at room temperature produced optimal values of t_{st} = 2.90 months and C5 concentration = 0.76% (w/v).

Two chitosan samples with close M_w values (~34 kDA and ~28 kDa) were obtained through of a KIO_4-depolymerization process applied to a commercial sample of chitosan. The potential stimulant activity of these chitosan samples on germination of two-layers coated zucchini seeds was studied. A markedly superior effect was found only for the chitosan sample containing a higher fraction of oligomeric chains, demonstrating that studied oligo-chitosans have noticeable germination-stimulating properties on zucchini seeds. These results seem to provide high value support to recent proposals considering chitosan oligomers as belonging to the oligosaccharins, a new group of plant growth promoter.

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