

# TEMPERATURE-DEPENDENT GERMINATION AND ENDO- $\beta$ - MANNANASE ACTIVITY IN SESAME SEEDS†

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**ABSTRACT** – The effects of temperature on germination and endo- $\beta$ -mannanase activity in seeds of *Sesamum indicum* was investigated. The minimum germination temperature (Tmin) lies between 12.8°C and 13.2°C while the maximum temperature (Tmax) is located between 45.5°C and 46°C. Germinabilities are statistically not different from estimated viability (88%) between 18.8°C and 43.2°C. The Mann-Whitney test indicated the interval 31.9°C to 35.1°C as the optimum temperature (Topt) range for germination rate. When seeds incubated at temperatures at or below the Tmin and close to or above the Tmax were transferred to 30°C, those incubated at lower temperatures achieved high germinability. On the other hand, the higher the pre-incubation temperature above Tmax, the lower the germinability achieved near Topt. Seed endosperm cell wall was found to contain mannose as the main monosaccharide. An increase in endo- $\beta$ -mannanase activity in the micropylar endosperm prior to seed germination was observed only at supra-optimum temperature.

**ADDITIONAL INDEX TERMS:** endosperm, germination rate, sesame

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## EFEITO DA TEMPERATURA NA GERMINAÇÃO E NA ATIVIDADE DA ENDO- $\beta$ -MANANASE EM SEMENTES DE GERGELIM.

**RESUMO** - O efeito da temperatura sobre a germinação e a atividade de endo- $\beta$ -mananase em sementes de *Sesamum indicum* foi investigada. A temperatura mínima de germinação (Tmin) está localizada entre 12,8°C e 13,2°C enquanto a temperatura máxima (Tmax) encontra-se entre 45,5°C e 46°C. As germinabilidades (G%) não foram significativamente diferentes da viabilidade estimada (88%) entre 18,8°C e 43,2°C. O teste de Mann-Whitney apontou o intervalo de 31,9°C a 35,1°C como sendo a faixa de temperatura ótima (Tot). Quando sementes incubadas a temperaturas próximas ou abaixo de Tmin e próximas ou acima de Tmax foram transferidas para 30°C, aquelas submetidas a baixas temperaturas atingiram germinabilidade elevada. Por outro lado, quanto maior a temperatura de pre-incubação acima de Tmax, menor a germinabilidade alcançada. O principal monossacarídeo encontrado na parede celular do endosperma das sementes foi manose. Somente em temperatura supra-ótima foi observada elevação na atividade de endo- $\beta$ -mananase na região micropilar do endosperma anterior à germinação.

**TERMOS ADICIONAIS PARA INDEXAÇÃO:** endosperma, gergelim, velocidade de germinação

### INTRODUCTION

Seed germination, by definition, starts with water imbibition by the dry seed and ends with radicle emergence (Bewley and Black, 1994). In many seeds, the tissues surrounding the embryo form a barrier that needs to be overcome in order for germination to occur. Recent evidence has shown that some seeds can do this through the enzymatic attack on these tissues (Black, 1996). Weakening of the tissue adjacent to the radicle tip (micropyla) precedes radicle emergence in seeds of *Lycopersicon esculentum* (tomato) (Groot et al., 1988; Nomaguchi et al., 1995), *Lactuca sativa* (lettuce) (Dutta et al., 1997), *Capsicum annuum* (pepper) (Watkins et al., 1985), *Datura ferox* (fierce thornapple) (Sánchez and de Miguel, 1997), *Picea glauca* (white spruce) (Downie et al., 1997) and *Nicotiana tabacum* (tobacco) (Leubner-Metzger et al., 1995). Most of these seeds have a mannan rich endosperm and evidence suggests that endo- $\beta$ -mannanase plays a role in the degradation of its cell walls associated with tissue weakening.

Several studies have evaluated endo- $\beta$ -mannanase activity during seed germination (Black, 1996). Although a few have correlated such activity with germination temperatures (Leviatov et al., 1995; Dahal et al., 1997; Dutta et

al., 1997; Nascimento et al., 2000), up to now, at least to our knowledge, only one has evaluated supra-optimum temperature effect on endo- $\beta$ -mannanase activity prior to seed germination (Nascimento et al., 2000). On the other hand, some studies with lettuce seeds showed no endo- $\beta$ -mannanase activity in the endosperm before germination (Halmer et al., 1976; Nonogaki and Morohashi, 1999).

In order to study the effect of temperature on endo- $\beta$ -mannanase activity during seed germination, it is first necessary to study its effect on seed germination. The temperature below which germination does not occur is referred to as the minimum germination temperature (Tmin) and that above which germination is inhibited is the maximum germination temperature (Tmax). Within this range there is an interval in which germination rate is highest (Topt) (Labouriau, 1972, 1978).

*Sesamum indicum* L. (sesame, commonly known in Brazil as "gergelim") is an oilseed cultivated in all regions of Brazil, having special importance in the Northeast region where it was introduced as an alternative to cotton. Sesame seeds are cultivated mainly for use in food industries and restaurants and for oil production (Beltrão et al., 1994). They contain approximately

50% oil, rich in unsaturated fat (Chung et al., 1995). The fat, stored as triacylglycerols (TAG) in intracellular organelles called oil bodies located in the cotyledons, is used as energy source for germination and postgerminative seedling growth (Huang, 1992; Tzen et al., 1997). Lipid content drops quickly during germination, over 85% of the TAG is depleted within four days (Kim, 1983; Tzen et al., 1997). According to Kim (1983), the rate of decrease is lower at 15°C than at 25°C.

During preliminary experiments with black sesame seed germination, a dark colored drop was observed in the micropylar region prior to radicle emergence. This drop was also observed at low temperatures, where germination does not occur. These observations suggested cell wall rupture caused by hydrolytic activity. This work reports the determination of *S. indicum* seed endosperm cell wall composition, the characteristics of seed germination at different temperatures and the temperature- and time-dependent activity of the enzyme endo- $\beta$ -mannanase in an attempt to correlate this activity with seed germination parameters.

## MATERIAL AND METHODS

### Plant Material

Seeds of *S. indicum* of the black variety were harvested in the state of Goiás, center-west region of Brazil, and stored at 4 °C. Viability was estimated on a sample of 100 seeds using a 0.5% solution of 2,3,5 tri-phenyl tetrazolium salt (Copeland, 1976). Seed water content was estimated according to Justice (1972), using 10 samples of 20 seeds. A sample of 200 seeds was used to estimate their average fresh weight.

Germination experiments were carried out in a thermogradient block (Labouriau and Cavalcanti, 1996) regulated to provide a temperature interval from 10°C to 50°C. Four replicates of 50 seeds were used for each temperature treatment. The seeds were placed in plexiglass plates with two layers of qualitative filter paper (Whatman, Springfield Mill, England).

The plates were put inside Pyrex tubes, which were closed with corks and placed inside the stations. Observations were made at 4 h or 8 h intervals, according to the germination rate. At each observation, germinated seeds were counted and removed and deionized water was added when necessary to guarantee maximum humidity. Seeds not germinated at extreme incubation temperatures were transferred to 30°C. Viability of seeds which did not germinate at 30°C was evaluated using tetrazolium solution.

Quantitative analysis of the results was done according to Labouriau (1972, 1978). Germinability  $[G\%=(\sum ni.N^{-1}).100]$ , average germination time  $(\bar{t}=\sum ni.ti.\sum ni^{-1})$  and its variance  $[S^2_v=\sum ni.(ti-\bar{t})^2.(-1+\sum ni^{-1})]$  average germination rate  $(v=\bar{t}^{-1})$  and its variance  $[S^2_v=(v)^4.S^2_t]$  (where  $i$  is the number of hours after the start of incubation,  $n_i$  is the number of germinated seeds between the observations made at  $t_{i-1}$  and  $t_i$  and  $N$  is the number of seeds used in each treatment) were calculated. Germinability confidence intervals were estimated using Tablas Cientificas (Documenta Geigy, 1965), with an  $\alpha = 0.05$ .  $T_{min}$  and  $T_{max}$  were identified as the temperatures in which germinability respectively ceases or starts to be significantly different from zero (Labouriau, 1984). Isothermal germination rate variance homogeneity was tested using Bartlett's test ( $\alpha = 0.05$ ). Isothermal germination rates were compared using Kruskal-Wallis's test ( $\alpha = 0.05$ ) followed by Mann-Whitney's test, with  $\alpha = 0.1$  (Sokal and Rohlf, 1995). The group of highest germination rates with non-significant differences was defined as the  $T_{opt}$  range (Labouriau, 1978).

Cell wall polysaccharide extraction was adapted from Gorshkova et al., (1996), since the cell wall was very resistant to extraction, even at a high alkali concentration. Seeds were boiled for 30 sec and their endosperms removed and dried. After incubation at 80°C for 15 min, they were homogenized in distilled water at 9,500 rpm for 2 h (Ultra Turrax T25, IKA Lab.) and filtered through nylon. The residue was sonicated in distilled water and extracted in 1 M NaOH solution containing sodium borohydrate ( $NaBH_4$ ) for 16 h. After

filtration, the residue was extracted for 24 h in 4 M NaOH solution containing  $\text{NaBH}_4$  and re-extracted for 18 h. The filtrates were combined and neutralized with HCl. Precipitation occurred during neutralization. After centrifugation at 12,000 g for 30 min at 10°C, the supernatant (SB) and sediment (SD) were dialyzed and lyophilized.

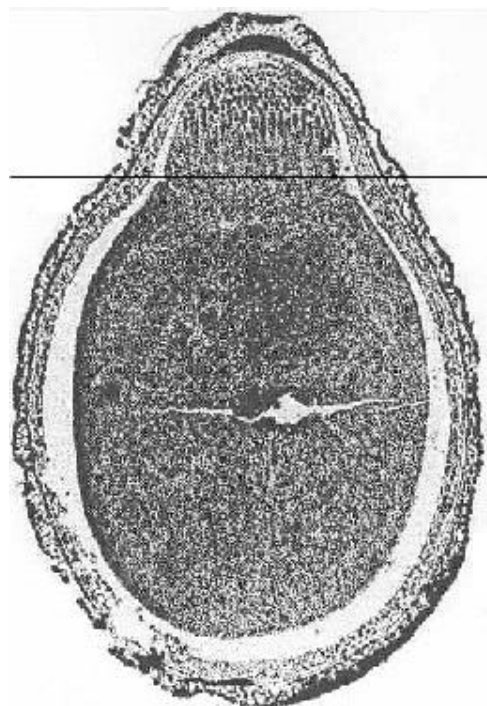
Acid hydrolysis of the extracted polysaccharides SB and SD was carried out according to Saeman *et al.*, (1945). The monosaccharides produced were detected by HPAEC-PAD (Dionex) using isocratic elution (23 mM NaOH, 0.8 mL.min<sup>-1</sup>) for 20 min with a PA-1 column (CARBOPAK). Proportion of the monosaccharides were corrected according to the detector sensitivity to each monosaccharide, calculated by using an equimolar standard.

Based on the germination parameters obtained, an infra-optimum temperature near  $t_{min}$ , a temperature located in the optimum range and a supra-optimum temperature near  $t_{max}$ , all of them located in the maximum germinability range but with different germination rates were selected. At each temperature, time-dependent activity of endo- $\beta$ -mannanase in the endosperm was investigated until 10% seed germination (20°C and 43°C) and 35% seed germination (34°C), in at least eight different periods of time after the start of incubation. The following temperatures and periods of time were chosen: 20°C for 0, 8, 16, 24, 32, 40, 48, 56 and 64 h; 34°C for 0, 2, 4, 6, 8, 10, 12 and 14 h; 43°C for 0, 4, 8, 12, 16, 20, 24, 28 and 32 h. It should be noted that 0 h corresponded to 20 min of imbibition.

The endosperm was removed from 100 ungerminated seeds submitted to the different treatments and separated in micropylar region (ME, corresponding to the endosperm adjacent to the radicle tip, measuring approximately 0.5 mm) and lateral region (LE, corresponding to the remaining endosperm) (Fig. 1), homogenized at 8,000 rpm for 2 min (Tissue Tearor, Biospec Products Inc.) in 1 ml ice cold extraction buffer (0.1 M citrate/0.2 M sodium phosphate buffer pH 7.0) and centrifuged at 13,500 g for 15 min at 4°C

(Voigt and Bewley, 1996). Protein content was determined according to Sedmak and Grossberg (1977).

The supernatants were assayed viscometrically for endo- $\beta$ -mannanase activity. The assay mixtures, consisting of 100  $\mu\text{L}$  of the supernatant (extract of ten endosperms) and 500  $\mu\text{L}$  of a 0.85% solution of galactomannan (Sigma, St. Louis, USA) in 0.1 M citrate/0.2 M sodium phosphate buffer pH 5.0 as substrate, were incubated for 20 min at 40°C. Changes in solution viscosity were measured every 4 min by timing its flow through a 0.2 mL glass pipette between marks made at 0 and 0.1 mL. Samples were assayed in triplicate. Control incubations were made with extraction buffer. The rate of decrease in viscosity was linear for the first 12 min. Enzyme activity was expressed in viscometric unit (Uvis), defined as the slope of the decrease in flow time during the first 12 min, per mg protein.



**FIGURE 1** - Longitudinal cut of a sesame seed indicating where seed was separated in micropylar tip and lateral part.

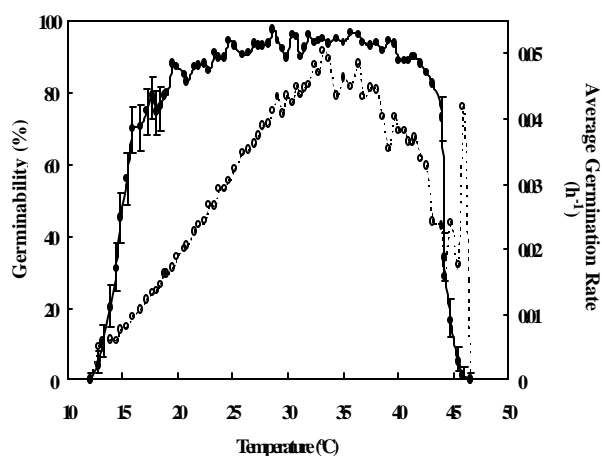
## RESULTS

Seed viability was estimated as 88% according to the tetrazolium test. Seed average fresh weight was calculated as  $2.47 \pm 0.53$  mg. Kolmogorov-Smirnov's test ( $\alpha = 0.01$ ) showed that seed fresh weight follows a normal distribution, thus indicating the homogeneity of the sample (Sokal and Rohlf, 1995). Seed water content was estimated as 5.47%, characterizing this seed as orthodox.

Figure 2 shows the germinability and germination rate of *S. indicum* seeds under different temperature treatments. Statistical analysis of the results pointed out the following germination parameters:  $T_{min}$  lies between  $12.8^{\circ}\text{C}$  and  $13.2^{\circ}\text{C}$ , while  $T_{max}$  is located between  $45.5^{\circ}\text{C}$  and  $46^{\circ}\text{C}$ . Germinabilities are statistically not different from the tetrazolium estimated viability (88%) between  $18.8^{\circ}\text{C}$  and  $43.2^{\circ}\text{C}$ . Statistical analysis of the germination rates established  $T_{opt}$  between  $31.9^{\circ}\text{C}$  and  $35.1^{\circ}\text{C}$ .

Seeds incubated for 480 h at temperatures near or below  $T_{min}$  (72 h after germination ceased between  $10^{\circ}\text{C}$  and  $15^{\circ}\text{C}$ ) and for 152 h at temperatures close to or above  $T_{max}$  (40 h after germination ceased between  $45^{\circ}\text{C}$  and  $50^{\circ}\text{C}$ ) were subsequently transferred to  $30^{\circ}\text{C}$ . Germinabilities before and after transfer as well as germination rates at  $30^{\circ}\text{C}$  are shown in tables 1 and

2. Low temperatures were not deleterious to the seeds, after being transferred to  $30^{\circ}\text{C}$  they achieved high germinability. On the other hand, seeds incubated at temperatures near or above  $T_{max}$  were differently affected: the higher the temperature the lower the germinability and the germination rate after transfer.



**FIGURE 2** - Effect of temperature on the germinability (●) and germination rate (○) of *Sesamum indicum* seeds. The bars represent 95% confidence intervals of germinability, but are absent at the points where germinability does not differ from 88% (tetrazolium estimated viability).

**TABLE 1** - Germinability of *Sesamum indicum* seeds incubated at temperatures below  $T_{min}$  for 480 h and after transfer to  $30^{\circ}\text{C}$  and germination rate after transfer.

Average Incubation Temperature ( $^{\circ}\text{C}$ )	Germinability at Incubation Temperature (%)	After Transfer to $30^{\circ}\text{C}$	
		Germinability (%) (number of seeds)	Germination Rate* ( $\text{h}^{-1}$ ) ( $\times 10^{-2}$ )
10.0	0	69.3 (163)	4.06a
10.6	0	75.3 (166)	4.13a
11.0	0	75.1 (169)	4.01a
11.5	0	79.2 (173)	4.02a
12.1	0	68.8 (154)	3.91a
12.8	4	63.0 (146)	3.91a
13.2	10.5	58.9 (146)	4.03a

\* Different letters indicate statistical difference according to Student's modified test ( $P < 0.05$ ).

**TABLE 2** - Germinability of *Sesamum indicum* seeds incubated at temperatures near T<sub>max</sub> for 152 h and after transfer to 30°C and germination rate after transfer.

Average Incubation Temperature (°C)	Germinability at Incubation Temperature (%)	After Transfer to 30°C	
		Germinability (%) (number of seeds)	Germination Rate* (h <sup>-1</sup> ) (x10 <sup>-2</sup> )
44.8	16.5	59.0 (161)	3.09b
45.5	5	49.7 (189)	2.11c
46.0	1	18.6 (188)	2.80bc
46.6	0	2.5 (200)	0.46d

\* Different letters indicate statistical difference according to Student's modified test (P < 0.05).

The polysaccharide present in sesame seed endosperm cell wall was extremely insoluble in water and could only be extracted with 4 M NaOH. Cell wall polysaccharide composition of both SB and SD samples were similar (Table 3). The main monosaccharide identified in both samples was mannose. Lower amounts of arabinose, galactose, xylose and glucose were also detected.

When enzyme activity was present, the flow time x incubation time curve showed a steep initial drop, stabilizing between 12 and 20 min (data not shown). A basal enzyme activity was observed at all the temperatures tested. At 20°C and 34°C, no significant rise in endo-β-mannanase activity was detected prior to seed germination (Figs. 3A and 3B). However, at 43°C, a sharp increase in enzyme activity was detected in the

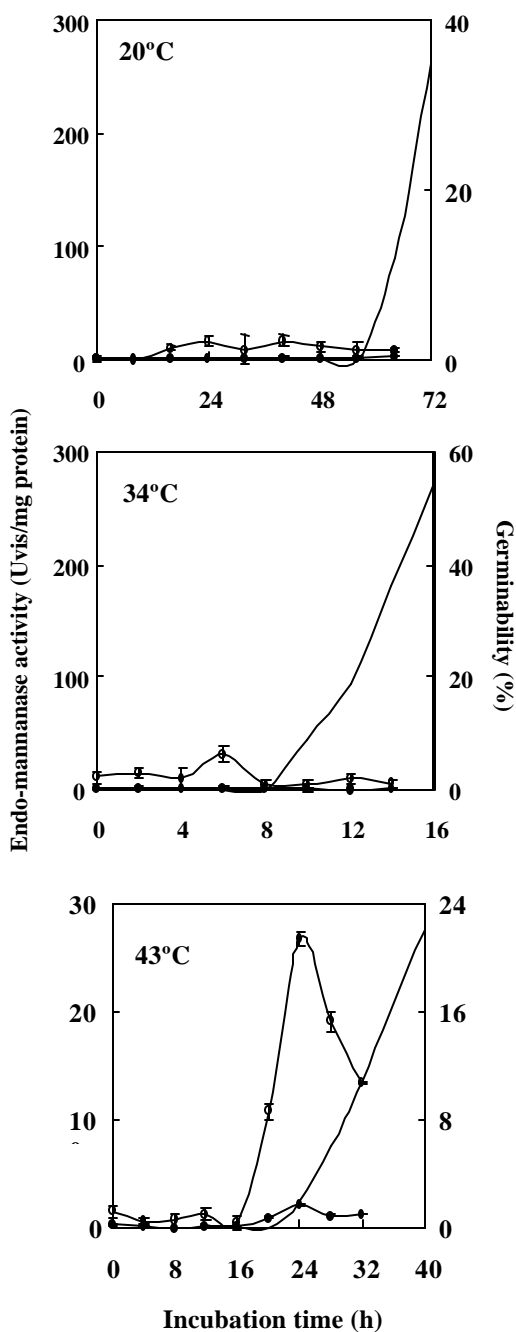
micropylar region of the endosperm after 16 h, reaching a peak at 24 h. There was no significant rise in enzyme activity in the lateral region (Fig. 3C).

## DISCUSSION

The large temperature range in which *S. indicum* seed germinates is a characteristic which may contribute to the wide distribution of this species in various regions of the world (Purseglove, 1968). In this interval, the uniformity of the germinability contrasts with germination rates variability. As shown in figure 2, germination rate rises linearly with increasing temperatures in the infra-optimum germination interval and decreases as temperature rises in the supra-optimum interval, in agreement with results obtained from experiments with seeds from other species (Roberts, 1988; Lima *et al.*, 1997).

**TABLE 3** - Monosaccharide composition of the supernatant (SB) and the sediment (SD) of the cell wall polysaccharide extract from *Sesamum indicum* seed endosperm. Composition as % of the total monosaccharides detected by HPAEC-PAD (Dionex).

Extract	Monosaccharide Composition (%)					
	Mannose	Galactose	Arabinose	Xylose	Glucose	Rhamnose
Supernatant	83.80	1.86	5.17	4.17	4.53	0.48
Sediment	82.48	2.46	11.48	0	3.58	0



**FIGURE 3** - Endo- $\beta$ -mannanase activity in extracts from lateral region, LE, (●) and micropylar region, ME, (○) of ten sesame seed endosperms until 10% germination (20°C and 43°C) and 35% germination .

Table 1 shows that seeds incubated at temperatures below  $T_{min}$ , after being transferred to 30°C, achieved high germinability within the first 24 h. Kim (1983) obtained similar results for seeds of different sesame varieties. On the other hand, the higher the incubation temperature the lower seed germinability and germination rate after transfer to 30°C (Table 2). Germination at temperatures below but near  $T_{max}$  was low, however transfer of non-germinated seeds to  $T_{opt}$  showed that a few were still viable. Studies with deuterium oxide have shown that temperature-dependent seed germination may be a reflection of temperature effect on protein structure (Labouriau, 1977, 1980). Cotyledon emergence opposite the micropylar region was observed in approximately 2% of the germinated seeds (data not shown), indicating that this region can become a barrier and suggesting the involvement of specific hydrolytic enzymes in its degradation.

The main monosaccharide found in the endosperm cell wall of *S. indicum* seeds was mannose, suggesting a mannan rich endosperm (Table 3). Endo- $\beta$ -mannanase activity has been detected in the endosperm of different seeds prior to radicle emergence (Black, 1996). A relationship between this activity and germination temperature has been established (Leviatov et al., 1995; Dahal et al., 1997; Dutta et al., 1997; Nascimento et al., 2000). In order to verify whether temperature effect on sesame seed germination could be correlated with its effect on endo- $\beta$ -mannanase activity in the endosperm, this enzyme was monitored until 10% germination (20°C and 43°C) and 35% germination (34°C) at temperatures providing different germination rates and maximum, comparable germinabilities.

The steep initial drop observed in the viscosity assays is typical of endo- but not of exopolysaccharidase activity (Reid et al., 1977). As previously described by Halmer et al., (1976) and Leviatov et al., (1995), there is a basal level of endo- $\beta$ -mannanase activity present even after 20 min of imbibition (Fig. 3,  $t = 0$ ), not evident in the lateral endosperm because of the scale used. This

basal activity is higher in the micropylar region at all the temperatures analyzed (Fig. 3). At sub-optimum (20°C) and optimum (34°C) temperatures, no rise in endo- $\beta$ -mannanase activity was detected prior to seed germination (Figs. 3A and 3B). These results disagree with previous studies that showed an increase in mannanase activity prior to tomato seed germination at sub-optimum temperatures (Leviatov *et al.*, 1995; Dahal *et al.*, 1997) and a rise in endo- $\beta$ -mannanase activity in cell wall extracts of lettuce endosperm before radicle emergence at optimum temperature (Dutta *et al.*, 1997). At 43°C, a supra-optimum temperature, endo- $\beta$ -mannanase activity began to increase in the micropylar endosperm after 16 h of seed incubation, while germination began only after 20 h (Fig. 3C). Rise in endo- $\beta$ -mannanase activity prior to seed germination at supra-optimum temperature has also been detected in lettuce (Nascimento *et al.*, 2000).

The results obtained suggest that endo- $\beta$ -mannanase is a constitutive enzyme, since its activity could be detected even after 20 min of imbibition at all temperatures tested. However, an increase in endo- $\beta$ -mannanase activity is both temperature- and time- dependent. It was observed after 16 h of sesame seed incubation at 43°C but not at 20°C. At 34°C, almost 40% of the seeds had germinated after 14 h but there was no rise in endo- $\beta$ -mannanase activity, contradicting previous findings for other seeds. The results point to the conclusion that increased enzyme activity is not required for germination to occur at sub-optimum (20°C) and optimum (34°C) temperatures. As suggested by Nascimento *et al.*, (2000) for lettuce, sesame seed germination at these temperatures may require lower endo- $\beta$ -mannanase activity. In this case, the basal level of activity observed in the micropylar region would be enough for germination to occur. A rise in endo- $\beta$ -mannanase activity was detected in the endosperm at the micropylar region before germination at supra-optimum temperature. Although Nascimento *et al.*, (2000) have shown a relationship between lettuce

seed germination at high temperature and an increase in endo- $\beta$ -mannanase activity before radicle protrusion, more studies are required to determine whether this activity is essential for sesame seed germination at high temperatures.

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