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Changes in germination and glyoxylate and respiratory enzymes of *Pinus pinea* seeds under various abiotic stresses

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Abstract
This study examined *Pinus pinea* seeds for their tolerance to osmotic potentials of −0.30 MPa (10% polyethylene glycol [PEG]), −0.58 MPa (18% PEG), −0.80 MPa (21% PEG), −1.05 MPa (24% PEG), pH values of 4, 5, 6, 7, 8, 9, 10, and different calcareous solutions (5, 10, 20 and 40% $CaCO_3$). The main enzymes of glyoxylate cycle and respiratory pathway were tested. *Pinus pinea* seeds under no stressful condition (Control) and 5% $CaCO_3$ reached 100% of germination. Higher concentrations of $CaCO_3$ (20, 40%) and lower pH (4–5) adversely affected seed germination percentage, glyoxylic and respiratory enzyme activities. PEG caused the most detrimental effects on *Pinus* seeds; increasing the osmotic potential the germination was completely inhibited. These results suggest that *Pinus pinea* is able to germinate in calcareous and alkaline soils rather than in soils with lower water availability and acidic conditions.

Keywords: *Abiotic stress, conifers, glycolysis, glyoxylate cycle,* Pinus pinea*, seed germination*

Introduction

Pinus pinea L. (Italian stone pine), is a timber species native of the Mediterranean river basin. It is used for timber, resin production construction purposes, furniture making and to a lesser extent for the pulp and paper industry. Their seeds are used as nourishment, so it is an important plant species with great economic, ecological, recreational and landscape value. It is widely distributed all over the Mediterranean region. It was probably disseminated as early as the first human explorations (Fallour et al. 1997).

Pine populations in Italy are mostly artificial; they were introduced at the beginning of 20th century without knowing the seed origin, particularly to restore disturbed forest areas. In Italy, *Pinus pinea* is the most planted species along the Mediterranean coast line over sandy, porous soils for sand dune stabilization, or over soils derived from calcareous bedrock (Varol & Tatli 2002).

On the sandy coast soils, the environmental stresses are promoted mainly by the lower water availability that reduce germination, limiting water absorption by seeds (Dodd & Donovan 1999) and affecting the mobilization of stored reserve (Lin & Kao 1995, Almasouri et al. 1999). In soil derived from calcareous bedrock, the primary limitation is promoted by direct toxic effects of high soil calcium concentration and by the high pH (8.0–8.5), characteristic of the solution of calcareous sites that could interfere directly with enzymes involved in the germination process, affecting their catalytic activity.

Plants adapt to stress by different mechanisms, including changes in morphological and developmental patterns as well as physiological and biochemical processes (Bohnert et al. 1995). Adaptation to all these stresses is associated with metabolic adjustments that lead to the accumulation of organic solutes such as sugar, polyols, betaines and proline (Flowers et al. 1977, Gorham et al. 1981). Among these accumulating solutes, starches and lipids give rise to sugars during seed germination and these are transported to sites where they are required for germination and growth (Koster & Leopold 1988, Gill et al. 2003).

Germination is the most vulnerable stage in the life cycle of plants and determines when and where seedling growth begins (Kigel 1995). Therefore, appropriate germination responses of species to environmental parameters determine their distribution in the environment. In particular, in the seeds reaching maturity in a highly dehydrated state, the orthodox seeds (Roberts 1973) germination is

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characterized by dramatic change in metabolism which allows the degradation of stored macromolecules and the recovery of the biosynthetic processes necessary for efficient germination. In coniferous species (oilseeds) a specialized metabolic pathway 'glyoxylate cycle' plays a central role in the use of stored oil, converting stored lipid reserves to glucose, the main respiratory substrate necessary to germination (Meletou-Christou et al. 1990).

One of the major changes during germination is a rapid increase in respiration. This involves glycolysis, the oxidative pentose phosphate pathway (OPPP), the tricarboxylic cycle and oxidative phosphorylation. Neither the relative importance of these pathways, nor the contribution of each enzyme to the regulation of these pathways as a whole, is as yet fully understood (Botha et al. 1992). However, it is likely that increased respiratory activity in seeds is linked to increased glycolytic activity (Podestà & Plaxton 1994). The OPPP pathway takes substrate from glycolysis, and feeds its products back into glycolysis, so the activity of this pathway is also important in determining the flux through glycolysis.

In natural field conditions, the capacity of the plant to achieve its whole cycle is directly related to its ability to recover after stress period. Data on recovery properties are available for whole plants (Rigolot 2004, Dominguez-Lerena et al. 2006); only few data are available on the behaviour of *Pinus pinea* seeds in this respect.

Stone pine is commonly considered the symbol of Italian coastal forests; consequently, its conservation is one of the most important objectives of current management strategies. Therefore, in this study we present details on germination and status of glyoxylate and respiratory enzymes involved in the first steps of germination of *Pinus pinea* seeds under various abiotic stresses.

Materials and methods

Seed treatment

Seeds of *Pinus pinea* L. were randomly collected from *Pinus pinea* stands in Castrovillari (Calabria Apennines). Healthy and uniform seeds of *Pinus pinea,* removed from the cones were surface sterilized for 10 min in 10% sodium hypochlorite solution, rinsed three times with sterile bidistilled water and deprived of their lignified tegument (free seeds).

Twenty free seeds were then transferred to sterile 13 cm Petri dishes containing two Watman's No. 1 filter paper moistened with 4.5 ml of bidistilled water, (Control) or with different concentrations of polyethylene glycol (PEG-6000). This osmotic agent was used in four iso-osmotic concentrations esteemed by a Wescor vapour pressure osmometer (Model 5500) and corresponding to final osmotic potentials of -0.30 MPa (10% PEG), -0.58 MPa (18% PEG), -0.80 MPa (21% PEG), and -1.05 MPa (24% PEG).

As regarding calcareous and pH treatment, 20 seeds of *Pinus pinea*, were placed in sterile 13 cm Petri dishes containing two Watman's No. 1 filter paper moistened with 4.5 ml of bidistilled water (Control) or with different concentrations of $CaCO_3$ (5, 10, 20, 40%). The bidistilled water were acidified at pH 4, 5, 6 and 7 using 0.01 M HCl and alkalinized at pH 8, 9, 10 using 0.01 M KOH.

Five replicates of each treatment were incubated in a germination chamber, using a regime of 16 h light/8 h darkness, 24°C and a relative humidity of 70%. Germination percentage was recorded daily up to 72 h using radicle extrusion (>2 mm long) as criterion. A seed was considered to show abnormal germination if shoot growth occurred in the absence of radicle extrusion. Fresh weight (FW) of seeds was recorded at 72 h from the start of the trial. For dry weight (DW) determination, samples were dried at 70°C in an oven until there was no decrease in weight. Relative water content (RWC) was also measured and expressed as a percentage according to the following equation:

$$\mathrm{RWC}\ (\%) = (\mathrm{FW} - \mathrm{DW}/\mathrm{FW}) \times 100$$

A set-up of 20 seeds for each treatment formed an experimental block.

Enzyme extraction and assay conditions

On the germination final days, for glyoxylate enzyme activities, seeds were homogenized (1:10 w/v), using a chilled pestle and mortar, with a 0.1 M K-phosphate buffer (pH 7.6) containing 10 mM $MgCl_2$, 1 mM Na_2-EDTA, 1 mM dithiothreitol (DTT). The extracts were centrifuged at 20,000 ***g***, for 20 min. The supernatant was used for glyoxylate enzyme analysis. All steps were performed at 4°C.

Phosphoenolpyruvate carboxykinase (PEPCK EC 4.1.1.49) was determined in the direction of oxaloacetate formation. The assay contained in 1 ml, 100 mM Hepes (pH 7.0), 10 mM $MnCl_2$, 100 mM $KHCO_3$, 2 mM glutathione (reduced form), 0.2 mM NADH, 10 mM Phosphoenolpyruvate, 24 units of L-malate dehydrogenase, and 50 µl extract. The consumption of NADH was determined by measurement of optical density at 340 nm ($\varepsilon = 6.22\ \mathrm{mM}^{-1}\ \mathrm{cm}^{-1}$) (Walker et al. 1995).

Malate synthase (MS EC 4.1.3.2), assay contained 140 mM K-phosphate (pH 7.5), 5 mM $MgCl_2$, 3.6 mM DTNB, 1 mM acetil-CoA, 3.3 mM Na-glyoxylate, 50 µl extract The activity was measured spectrophotometrically at A_{405}, ($\varepsilon = 13{,}6\ \mathrm{mM}^{-1}\ \mathrm{cm}^{-1}$) (Bajaracharya & Schopfer 1979).

Isocitrate lyase (ICL EC 4.1.3.1), assay mixture consisted of 80 mM Hepes (pH 7.0) 6 mM $MgCl_2$,

4 mM phenylhydrazine and 20 μl extract. The reaction was started by adding substrate (4 mM D,L-isocitrate). Glyoxylate-phenilhydrazone ($\varepsilon = 17\ mM^{-1}\ cm^{-1}$) formation was followed at 30°C at 324 nm (Ranaldi et al. 2003).

For cytrate synthase (CyS EC 1.11.1.6) activity determination to Tris-HCl 0.1 M (pH 8.0) were added 0.17 mM oxalacetic acid, 0.2 mM acetyl-CoA, and 10 μl extract. The activity was measured spectrophotometrically at A_{232} ($\varepsilon = 0.541\ mM^{-1}\ mm^{-1}$) (Bergmeyer et al. 1983a).

For respiratory enzyme activities, seeds were extracted with a mortar and pestle in 100 mM Hepes-NaOH pH 7.5, 5 mM $MgCl_2$, and 1 mM dithiothreitol (DTT), in a ratio 1:3 (p/v). The extract was filtered through two layers of muslin. The supernatant obtained by centrifugation at 20,000 ***g*** for 15 min was used for enzymatic analysis. All steps were performed at 4°C.

Glucokinase (GK, D-Glucose-6-phosphotransferase, EC 2.7.1.1) activity was measured by coupling hexose phosphate production with NAD reduction by glucose-6-phosphate dehydrogenase and monitoring at A_{340} ($\varepsilon = 6.22\ mM^{-1}\ cm^{-1}$) (Huber & Akazawa 1986).

Phosphoglucoisomerase (PGI, D-glucose-6-phosphate ketoisomerase, EC 5.3.1.9) assay contained 50 mM Hepes-NaOH (pH 7.2), 5 mM $MgCl_2$, 5 mM fructose-6-phosphate, 1 mM NAD, 1 IU ml^{-1} glucose-6-phosphate dehydrogenase. The activity was measured spectrophotometrically at A_{340} ($\varepsilon = 6.22\ mM^{-1}\ cm^{-1}$) (Tsai et al. 1970).

Pyruvate kinase (PK, EC 2.7.1.40) activity was determined adding 0.1 M triethanolamine (TEA) adjusted with 0.1 M NaOH to pH 7.75 3 mM β-NADH-Na_2 in 0.1 M TEA (pH 7.75), 52 mM adenosine 5′-diphosphate-Na_2 in 0.1 M TEA (pH 7.75), 0.15 M $MgSO_4$-$6H_2O$ and 0.15 M KCl in 0.1 M TEA (pH 7.75), L-lactic dehydrogenase and 50 μl extract. The reaction was started after a lag time of 10 min at 30°C by adding 0.225 M 2-phosphoenolpyruvate-Na-H_2O in 0.1 M TEA (pH 7.75) ($\varepsilon = 6.22\ mM^{-1}\ cm^{-1}$) (Bergmeyer et al. 1983b).

Phosphoenolpyruvate carboxylase (PEPc EC 4.1.1.31) enzymatic activity was spectrophotometrically measured by monitoring NADH oxidation at 340 nm for 5 min at 30°C. The assay medium contained 100 mM Tris-HCl pH 8.0, 10 mM $MgCl_2$, 10 mM $NaHCO_3$, 0.2 mM NADH, 1.5 U malic dehydrogenase (MDH) and 100 μl enzyme extract, ($\varepsilon = 6.22\ mM^{-1}\ cm^{-1}$) (Pasqualini et al. 2001).

Activity of Glucose-6-phosphate dehydrogenase (G6PDH EC 1.1.1.49) was measured spectrophotometrically by monitoring $NADP^+$ reduction at 340 nm at 30°C. The assay medium (1 ml) contained 50 mM Tris-HCl pH 8.0, 0.15 mM $NADP^+$, 10 mM $MgCl_2$, 2 mM glucose-6-phosphate and enzyme extract (Esposito et al. 2001).

Statistical analysis

The experimental design was a randomized complete-block design with five replications of each treatment. Data of germination were previously transformed so that distribution will be normal and the variances homogeneous. The transformation used was the arcsine of the square root of the proportion of germinated seeds per samples. Data were subjected to analysis of variance (ANOVA) and treatment means were compared using the Student-Newman-Keul test (Sokel & Rohlf 1969).

Results

Seed germination

The germination percentage of *Pinus pinea* seeds under different abiotic stresses ($CaCO_3$, pH and PEG) is shown in Table I.

Pinus pinea seeds under no stressful condition (control) reached 100% germination. Seed germination was differently affected by each stress.

Considering pH treatments, the greatest reduction of seed germination was observed at pH 4 (−50%) (Table I). Increasing the pH values the germination percentage progressively increased, reaching at pH 8, 9, and 10 a seed germination percentage of 95, 91 and 93%, respectively.

$CaCO_3$ treatments affected differently seed germination of *Pinus pinea* in response to the different doses. Seed germination percentage remained unaffected by 5% $CaCO_3$. 10% $CaCO_3$ reduced

Table I. Effect of different concentrations of $CaCO_3$ (5, 10, 20, 40%) and PEG (10, 18, 21, 24%), and different pH values (4, 5, 6, 7, 8, 9, and 10) on germination percentage of *Pinus pinea* seeds. Seed germination was measured 72 h after treatments.

Treatment	Germination%
Control (H_2O)	100^{a*}
$CaCO_3$ 5%	100^{a}
$CaCO_3$ 10%	80 $(2)^{c}$
$CaCO_3$ 20%	50 $(3)^{f}$
$CaCO_3$ 40%	10 $(1)^{h}$
pH 4	50 $(2)^{f}$
pH 5	62 $(3)^{e}$
pH 6	70 $(3)^{d}$
pH 7	74 $(4)^{d}$
pH 8	95 $(2)^{ab}$
pH 9	91 $(1)^{b}$
pH 10	93 $(2)^{b}$
PEG 10%	30 $(1)^{g}$
PEG 18%	0
PEG 21%	0
PEG 24%	0

*Value in the same column, followed by the same letter, are not statistically different at $p \leq 0.05$. Numbers in parentheses denote the standard error of the mean.

slightly seed germination percentage (−20%) in comparison to unstressed seeds. In contrast, the presence of 20 and 40% $CaCO_3$ drastically reduced *Pinus pinea* final germination percentage compared to control seeds (−50 and −90%, respectively, Table I). As regard osmotic treatments, seed germination was drastically reduced by 10% PEG (−70%); none of the seeds was able to germinate already at −0.58 MPa of PEG.

Relative water content of *Pinus pinea* seeds treated with different $CaCO_3$ concentrations started to decline at 20% $CaCO_3$ (−17% of control) and reached the minimum value (−60% of control) at 40% $CaCO_3$. A decrease in RWC of about 21–28%, with respect to control, was found at 6–4 pH, whereas RWC percentage was not significantly affected by 10–7 pH treatments. Osmotic stress (PEG) considerable reduced RWC (from 55–82%) (Table II).

Fresh and dry mass of seeds gradually decreased increasing $CaCO_3$ concentrations; the fresh and dry mass was also affected by pH treatments, with a greater reduction as the acidity increased. PEG was the most detrimental solute at all external osmotic potentials since it drastically reduced the increase in fresh and dry weight of germinating seeds comparatively to the other treatments (Table II).

Enzyme activities

The effects of different treatments on the enzyme activities in *Pinus pinea* seeds were variable with respect to concentrations used in various abiotic stresses.

Table II. Effect of different concentrations of $CaCO_3$ (5, 10, 20, 40%) and PEG (10; 18; 21; 24%), and different pH values (4, 5, 6, 7, 8, 9, 10) on fresh weight (FW, g), dry weight (DW, g) and water content [RWC (%) = (FW - DW/FW) × 100] of *Pinus pinea* seeds. These measures were recorded at 72 h from the start of experiments.

Treatment	FW	DW	RWC
Control (H_2O)	0.49 (0.003)^{a*}	0.26 (0.003)^{a}	46.93 (2.08)^{a}
$CaCO_3$ 5%	0.50 (0.005)^{a}	0.27 (0.003)^{a}	46.00 (1.38)^{a}
$CaCO_3$ 10%	0.44 (0.003)^{b}	0.24 (0.002)^{b}	45.45(2.15)^{a}
$CaCO_3$ 20%	0.28 0.001)^{e}	0.17 (0.001)^{e}	39.28 (1.28)^{b}
$CaCO_3$ 40%	0.22 (0.002)^{g}	0.18 (0.002)^{e}	18.18 (1.68)^{d}
pH 4	0.35 (0.002)^{d}	0.23 (0.003)^{bc}	34.28 (2.28)^{c}
pH 5	0.37 (0.003)^{d}	0.24 (0.003)^{a}	35.13 (1.89)^{c}
pH 6	0.40 (0.003)^{bc}	0.25 (0.002)^{b}	37.50 (2.04)^{bc}
pH 7	0.42 (0.003)^{b}	0.22 (0.002)^{c}	47.61 (3.00)^{a}
pH 8	0.50 0.002)^{a}	0.26 (0.003)^{a}	48.00 (3.02)^{a}
pH 9	0.49 (0.002)^{a}	0.27 (0.004)^{a}	46.89 (0.86)^{a}
pH 10	0.48 (0.004)^{a}	0.26 (0.003)^{a}	45.83 (1.27)^{a}
PEG 10%	0.29 (0.001)^{e}	0.23 (0.002)^{bc}	20.68 (3.05)^{d}
PEG 18%	0.24 (0.002)^{f}	0.21 (0.002)^{d}	12.50 (1.28)^{e}
PEG 21%	0.25 (0.001)^{f}	0.23 (0.003)^{bc}	8.00 (0.90)^{f}
PEG 24%	0.22 (0.001)^{g}	0.20 (0.002)^{d}	9.00 (1.25)^{f}

*Value in the same column, followed by the same letter, are not statistically different at $p \leq 0.05$. Numbers in parentheses denote the standard error of the mean.

Glyoxylate and respiratory enzyme activities were unaffected by 5% $CaCO_3$. A decline from 6–13% compared to unstressed seeds was observed in the presence of 10% $CaCO_3$. 20% $CaCO_3$ resulted in a marked reduction in the enzyme activities: PEP-CK and PEPc were the enzymes more affected by this treatment with a reduction of 56 and 60%, respectively, compared to control seeds. 40% $CaCO_3$ completely inhibited glyoxylic and respiratory enzymes (Tables III and IV).

As regarding the activities of glyoxylate and respiratory enzymes there were no significant difference between control and higher pH values (8–10). A significant and progressive decrease in these activities was observed in response to lower pH values (7–4). Among the glyoxylic enzymes, the ICL was more responsive to pH stress than the other three enzymes assayed (Table III).

PEG was the most detrimental solute; in fact, the enzyme activities recorded in the presence of PEG were always lower than those recorded in both control and other treatments. 10% PEG caused a drastically reduction (−66%) in the activities of glyoxylate and respiratory enzymes (Tables III and IV).

At higher osmotic potential values the activities of glyoxylate and respiratory enzymes were completely inhibited (Tables III and IV).

Discussion

The conservation of *Pinus pinea* forests in the Mediterranean region is a very complex task as

Table III. Effect of water (Control) and different concentrations of $CaCO_3$ (5, 10, 20, 40%), pH (4, 5, 6, 7, 8, 9, 10) and PEG (10; 18; 21; 24%), on isocitrate lyase (ICL), malate synthase (MS), phosphoenolpyruvate carboxykinase (PEP-CK), and cytrate synthase (CS) enzyme activities in *Pinus pinea* seeds. Enzyme activities are expressed in nkat-g^{-1} fresh weight.

Treatment	ICL	MS	PEP-CK	CS
Control (H_2O)	30 (1.1)^{a}	34 (3.6)5^{a}	23 (1.1)^{a}	37 (0.9)^{a}
$CaCO_3$ 5%	31 (0.9)^{a}	348 (2.9)^{a}	24 (1.3)^{a}	39 (1.3)^{a}
$CaCO_3$ 10%	26 (0.7)^{c}	298 (3.0)^{b}	20 (1.0)^{b}	32 (0.4)^{b}
$CaCO_3$ 20%	18 (1.0)^{e}	248 (2.9)^{cd}	10 (0.9)^{e}	26 (0.5)^{d}
$CaCO_3$ 40%	nd	nd	nd	nd
pH 4	13 (0.9)^{f}	235 (1.9)^{d}	16 (0.5)^{cd}	26 (0.3)^{d}
pH 5	18 (0.8)^{e}	260 (1.5)^{c}	15 (0.3)^{d}	27 (0.2)^{cd}
pH 6	20 (0.5)^{d}	280 (2.0)^{b}	17 (0.7)^{c}	29 (0.4)^{c}
pH 7	25 (0.3)^{c}	295 (2.0)^{b}	19 (1.0)^{b}	31 (1.0)^{b}
pH 8	29 (0.9)^{ab}	354 (2.2)^{a}	25 (1.1)^{a}	38 (0.7)^{a}
pH 9	28 (1.2)^{b}	350 (2,0)^{a}	24 (0.8)^{a}	37 (1.2)^{a}
pH 10	28 (0.9)^{b}	349 (1.9)^{a}	23 (0.5)^{a}	37 (0.9)^{a}
PEG 10%	10 (0.7)^{g}	105 (1.5)^{e}	9 (0.03)^{e}	11 (0.09)^{e}
PEG 15%	nd	Nd	nd	nd
PEG 18%	nd	Nd	nd	nd
PEG 21%	nd	Nd	nd	nd
PEG 23%	nd	Nd	nd	nd
PEG 26%	nd	Nd	nd	nd

*Value in the same column, followed by the same letter, are not statistically different at $p \leq 0.05$. Numbers in parentheses denote the standard error of the mean.

Table IV. Effect of water (Control) and different concentrations of PEG (10; 18; 21; 24%), $CaCO_3$ (5, 10, 20, 40%) and pH (4, 5, 6, 7, 8, 9, 10) on glucokinase (GK), phosphoglucoisomerase (PGI), phosphoenolpyruvate carboxylase (PEPc), pyruvate kinase (PK), and glucose-6-phosphate dehydrogenase (G6PDH). Enzyme activities are expressed in nkat-g^{-1} fresh weight. Values are means $\pm$SD of five replicates.

Treatment	GK	PGI	PEPc	PK	G6PDH
Control (H_2O)	84 (2.8)^{a}	0.90 (0.001)^{a}	28 (0.9)^{a}	87 (3.2)^{a}	46 (1.3)^{b}
$CaCO_3$ 5%	85 (2.5)^{a}	0.93 (0.009)^{a}	29 (1.1)^{a}	89 (3.0)^{a}	44 (0.9)^{bc}
$CaCO_3$ 10%	79 (1.9)^{b}	0.80 (0.001)^{b}	27 (1.2)^{a}	73 (1.8)^{c}	42 (1.1)^{c}
$CaCO_3$ 20%	71 (2.2)^{c}	0.62 (0.007)^{e}	11 (0.8)^{e}	46 (2.0)^{g}	30 (0.8)^{f}
$CaCO_3$ 40%	nd	nd	nd	nd	nd
pH 4	70 (2.5)^{c}	0.60 (0.006)^{e}	15 (1.0)^{d}	56 (1.3)^{e}	23 (0.7)^{h}
pH 5	77 (2.8)^{b}	0.68 (0.003)^{d}	16 (0.8)^{cd}	59 (1.1)^{e}	26 (0.5)^{g}
pH 6	78 (1.7)^{b}	0.79 (0.005)^{b}	18 (0.5)^{c}	69 (1.8)^{cd}	34 (1.0)^{e}
pH 7	77 (1.9)^{b}	0.80 (0.001)^{b}	20 (0.7)^{b}	71 (2.1)^{c}	39 (1.1)^{d}
pH 8	85 (2.5)^{a}	0.94 (0.002)^{a}	26 (0.2)^{a}	88 (1.8)^{a}	49 (1.5)^{a}
pH 9	78 (2.0)^{b}	0.89 (0.003)^{a}	27 (0.4)^{a}	79 (1.2)^{b}	47 (1.2)^{ab}
pH 10	79 (2.1)^{b}	0.75 (0.005)^{c}	27 (0.1)^{a}	67 (1.5)^{d}	45 (1.0)^{b}
PEG 10%	15 (1.8)^{d}	0.60 (0.006)^{e}	17 (0.7)^{c}	51(1.3)^{f}	24 (1.0)^{g}
PEG 15%	nd	nd	nd	nd	nd
PEG 18%	nd	nd	nd	nd	nd
PEG 21%	nd	nd	nd	nd	nd
PEG 23%	nd	nd	nd	nd	nd
PEG 26%	nd	nd	nd	nd	nd

*Value in the same column, followed by the same letter, are not statistically different at $p \leq 0.05$. Numbers in parentheses denote the standard error of the mean.

ecological conditions are not only highly variable among the countries but also extreme (low rainfall, high salinity, etc.). In this contest knowing *Pinus pinea* natural regeneration by seeds is a prerequisite to out limiting its potential distribution area.

The present investigation monitored changes caused by different abiotic stress in germination percentage, glyoxylate and respiratory enzyme activities of *Pinus pinea* seeds. Imposition of all stresses, except 5% $CaCO_3$, resulted in significant decreases in germination rate, but the maximum decrease occurred due to PEG, 20 and 40% $CaCO_3$ and pH 4 treatments.

The germination processes can be considered in terms of these sequential steps: imbibitions, metabolism leading to initiation of radicle growth, and radicle growth leading to radicle emergence. A threshold level of hydration is required for subsequent radicle elongation. Upon imbibitions, the quiescent dry seed rapidly resumes metabolic activity. The structures and enzymes necessary for this initial resumption of metabolic activity are generally assumed to be present within the dry seed. One of the first changes upon imbibitions is the resumption of respiratory activity, which can be detected within minutes. In fact, the rate of oxygen consumption by seeds increases during imbibitions with a further sharp increase in germination. The increase in oxygen consumption reflects the increased oxidation of carbohydrates, coming from the glyoxylate cycle, via the respiratory pathway. The OPPP operates alongside glycolysis, and the two pathways share a number of enzymes and intermediates (ap Rees 1980). It functions to provide the cell with NADPH for biosynthetic reactions and appears to be important in the regulation of germination (Come et al. 1988, Perino & Come 1991). The balance between glycolysis and the OPPP ensures that the seed is supplied with the necessary levels of reducing power, ATP and carbon skeletons for biosynthesis. In this work we have found that the failure of seeds to germinate, in presence of low water and pH values, is strongly correlated to the inhibition of glyoxylate pathway, that refund with glucose the glycolysis and the OPPP pathway. The central role of glyoxylate cycle in the oil seed germination process is due to the action of two enzymes ICL and MS that bypass the decarboxylation steps of the TCA cycle. Two moles of acetyl-CoA are introduced with each turn of the cycle, resulting in the synthesis of one mole of the four-carbon compound succinate that passes from the glyoxysome into the mitochondrion and enters the TCA cycle, where it is converted to malate. This malate is then exported to cytosol in exchange for succinate and is converted to oxalacetate. PEP ck catalyses the conversion of oxalacetate to phosphoenolpyruvate and this fuels the synthesis of soluble carbohydrates necessary to germination.

Water stress has been reported to limit the mobilization of reserves in several species (Lin & Kao 1995). Our results confirm these findings, showing in stressed seeds not only a decrease in enzyme activities of glyoxylate cycle, which operates in the conversion of fats to carbohydrates (Giachetti et al. 1987), but also a decrease in the enzyme activities of respiratory pathway. As regarding calcareous treatment, a comparison of the results obtained with those of the control seeds show that seeds exposure to higher concentrations of $CaCO_3$ cause a decrease or an inhibition of germination, probably

due to an increase in osmotic potential that cause a reduction in water absorption by seeds that limit the resumption of metabolic activity in seeds. Studies by Johanson et al. (1974) have shown that various monovalent and divalent anions have inhibitory effects on isocitrate lyase, the first enzyme unique to glyoxylate cycle. The type of inhibition was found to be competitive with respect to isocitrate, linear in the case of bivalents ions, and nonlinear in the case of monovalent ions. The authors Mackintosh and Nimmo (1988), Giachetti et al. (1988), and Ranaldi et al. (2000) suggested a probably interaction with two binding sites for magnesium in the active center of the enzyme. The intensity of decrease is related to ion concentrations. The results of this study carried out with *Pinus pinea* put in evidence a possible regulatory role of the glyoxylate cycle by inorganic anions in particular ($CO_3^{=}$). In addition, the decrease in germination observed in presence of solution with high concentration of $CaCO_3$ is not due to pH value itself, but rather to direct toxic effect of calcium or carbonate anions.

In the acidic solutions (pH 4.0 and 5.0) the direct toxicity of H^+ ions may be the possible cause of the poor seed germination. In conclusion, our study indicates that changes in levels of glyoxylate enzyme activities are among the first signals of seed response to stress conditions and show that *Pinus pinea* is able to germinate in calcareous and alkaline soils rather than in soils with low water availability and pH value.

The knowledge of this germinative behaviour is essential in order to understand the maintenance of undisturbed forests of *Pinus pinea* where the different abiotic stresses of Mediterranean environment, affect plant growth and productivity.

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