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**Cytosolic ascorbate peroxidase and Cu, Zn-superoxide dismutase
improve seed germination, plant growth, nutrient uptake and drought
tolerance in tobacco**

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Running title: Cytosolic antioxidant defenses, plant growth and germination

Abstract

1
2 The effects of over-expression of two cytosolic antioxidant enzymes (Cu,Zn-
3 SOD and/or APX) on plant nutrition, gas exchange, chlorophyll fluorescence,
4
5 SOD and/or APX) on plant nutrition, gas exchange, chlorophyll fluorescence,
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7 seed viability and germination in transgenic tobacco (*Nicotiana tabacum* cv.
8
9 Xanthi) under deficit irrigation or salinity conditions were investigated. Three
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11 transgenic lines of tobacco were used in this study: line 17, harboring 2 copies
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13 of the cytosolic CuZn-SOD (*cytsod*) gene; line 51, with 2 copies of the cytosolic
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15 APX (*cytapx*) gene and line 39, harboring one copy of each gene.
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19 Over-expression of cytosolic antioxidants enzymes in tobacco plants resulted in
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21 a better growth performance that correlated with an improved photosynthetic
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23 capacity and nutrient uptake. Moreover, *cytsod* or *cytapx* genes promoted seed
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25 germination, and enhanced tolerance to mild water stress. In addition, this
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27 enhanced antioxidant capacity protected seeds from ageing during prolonged
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29 storage, and stimulated germination under salt stress conditions. These results
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31 suggest that cytosolic antioxidant transgenes are useful tools to improve
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33 drought tolerance, nutrient uptake and seed germination under stressful
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35 conditions.
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43 **Key Words:** germination; plant growth; mineral nutrition; salinity; seed aging;
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45 seed physiology.
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1 Introduction

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2 Environmental stresses exert adverse effects on plant growth and
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4 development because of the reactive oxygen species (ROS) that are
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6 overproduced under these stressful conditions. Among them, anion superoxide
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8 ($O_2^{\cdot-}$), hydrogen peroxide (H_2O_2) and hydroxyl radicals ($\cdot OH$) are the most
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10 damaging ROS in plants (Perl-Treves and Perl 2002).
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14 Water and salt stress are serious stress factors that limit agricultural
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16 production in many regions worldwide (Alscher et al. 1997; Hernández et al.
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18 2001). Both, water deficit and salinity primarily affect photosynthetic CO_2
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20 assimilation leading to a restricted plant production (Flexas et al. 2002). This
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22 lower CO_2 availability favors the ROS generation in the chloroplast inducing an
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24 oxidative stress at cellular level (Asada ~~et al.~~ 1999; Faize et al. 2011). In
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26 addition, under drought and salt stress conditions ROS production also
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28 increased in other cell compartments such as mitochondria or peroxisomes
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30 (Alscher et al. 1997; Hernández et al. 1993; Mittova et al. 2003; Bartoli et al.
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32 2004).
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39 Germination can also induce ROS production (Hendry 1993; McDonald
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41 1999; Kranner et al. 2010), being traditionally considered as a negative effect.
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43 Seeds rapidly increase oxygen uptake and oxidative phosphorylation in order to
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45 support the energy for the germination process (Tommasi et al. 2001).
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47 Production of H_2O_2 has been demonstrated during the early imbibition stage of
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49 seeds of soybean (Puntarulo et al. 1991), tomato (Morahashi 2002), radish
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51 (Schopfer et al. 2001), wheat (Caliskan and Cuming 1998), and sunflower (Baily
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53 et al 2002). If ROS over-generation is not tightly controlled, they can cause
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1 detrimental effects during the initial phases of growth and development of
2 embryos and seedlings (Cakmak et al. 1993).
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4 Seeds can also be subjected to the detrimental effect of ROS production
5 during extreme desiccation and storage, and they can lose germination ability
6 and viability during prolonged storage periods (McDonald 1999). The excess of
7 ROS accumulation induces a reduced germination rate, which is considered as
8 an indication of seed ageing. Aged seeds loss plasma membrane integrity
9 (Priestley et al. 1985) which leads to an inability of cells to maintain osmotic
10 turgor, and therefore to seed death (Parrish et al. 1982). For these reasons,
11 antioxidative mechanisms have been regarded as being of particular
12 importance for the success of germination (de Gara et al. 1997; Tommasi et al.
13 2001). The antioxidant mechanisms are categorized into enzymatic and non-
14 enzymatic antioxidants. Non-enzymatic antioxidants include ascorbate (ASC),
15 glutathione (GSH), tocopherol, flavonoids, and carotenoids. The antioxidant
16 enzymes are important to cope with abiotic stress disorders in adult plants as
17 well as for the success of germination (Bailly ~~et al.~~ 2004; Barba-Espín et al.
18 2010). They include superoxide dismutase (SOD), the ASC-GSH cycle
19 enzymes, catalase and peroxidases that are involved in the scavenging of ROS
20 in plant cells (Noctor and Foyer 1998; Asada 1999). Among the antioxidative
21 enzymes, SODs and ascorbate peroxidases (APXs) play a pivotal role in ROS
22 detoxification in higher plants (Asada 1999). SODs are metalloenzymes that
23 catalyzes the dismutation of O_2^- to O_2 and H_2O_2 (Fridovich 1975). APXs, the
24 main enzyme of the ASC-GSH cycle, are one of the most important key
25 enzymes that scavenge potentially harmful H_2O_2 in different cell compartments
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(Noctor and Foyer 1998; Asada 1999; Jiménez et al. 1997; Diaz-Vivancos et al. 2006).

Beside their detrimental effect, ROS can also play a key role in the completion of germination and should be considered as a messenger or transmitters of environmental cues during seed germination (Bailly et al. 2008, Barba-Espín et al. 2010; 2011; Diaz-Vivancos et al. 2013). In recent works, it has been reported that the imbibition of pea seeds with low H₂O₂ levels increased the germination rate as well as the growth of the seedlings in a concentration-dependent manner, linked to the induction of some proteins related to plant signaling and development, cell elongation and division and cell cycle control, as well as to a strong decrease in ABA contents (Barba-Espín et al. 2010; 2011; Diaz-Vivancos et al. 2013). In *Arabidopsis thaliana*, germination was associated with an accumulation of superoxide and hydrogen peroxide in the radicle (Leymarie et al. 2012). In radish, germination was also accompanied with increase in ROS originated from seed coat and embryo (Oracz et al. 2009).

Previously, we transformed tobacco (*Nicotiana tabacum* cv. *Xanthi*) plants with cytosolic *Cu-ZnSOD* and/or *APX* genes. These transgenic lines displayed greater tolerance to mild water stress, by stimulating the antioxidant capacity in both the cytosol and the chloroplast (Faize et al. 2011). We extended this work to see if this higher antioxidative capacity can also be important to increase the germination of the derived T1 seeds and vigor of the tobacco seedlings. We also attempted to study the effect of these cytosolic transgenes in plant growth, mineral content, photosynthesis (V_{cmax} and J_{max} parameters) and seeds physiology under unfavorable environmental conditions.

2 Material and methods

2.1 Plant material, seed germination, plant vigor and biomass

Three transgenic lines of tobacco (*Nicotiana tabacum* cv. *Xanthi*) (T0 plants) were used in this study: line 17 harboring 2 copies of the cytosolic *Cu-ZnSOD* (*cytsod*) gene; line 51 with 2 copies of the cytosolic *APX* (*cytapx*) gene and line 39 harboring one copy of each gene. All of these cytosolic genes are under the control of the constitutive duplicated CaMV35S promoter (Faize et al. 2011). Transgenic and non-transgenic plants were grown in pots with peat substrate in the greenhouse under a 16/8 h day/night photoperiod at 25°C.

T1 seeds were collected from T0 plants 60 days after self-pollination at anthesis and used for further experiments. They were used immediately (new seeds) or stored for up to 4 years at 8°C (aged seeds). New seeds were germinated on Petri dish on three layers of filter paper disks soaked with 5 ml of sterile distilled water and incubated at 25°C up to 10 days.

In order to investigate the effect of ectopic expression of *cytsod* and *cytapx* genes on seed germination and plant growth vigor, seed germination rates were tested in non-transformed and transgenic T1 lines. Seeds were surface sterilized with diluted sodium hypochlorite solution (2%) for 5 min followed by three washes with sterilized distilled water. They were then germinated on three layers of filter paper disks soaked with 5 ml of sterile distilled water or with 5 ml of 100 mM NaCl solution. Seeds were incubated at 25°C with a 16/8 h photoperiod. The rate of germination was determined every 2 days by counting seeds with radicle emergence of at least 2 mm in length. The time course (measured in days) taken to reach 50% of germinated seeds was

1 also determined (T50). All assays were replicated four times using 50 seeds per
2 replicate.
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4 Growth parameters, such as roots and shoots length and shoots fresh
5 weight from germinated seeds were measured 14 days after seed imbibition,
6 and seedlings were transferred to pots with peat substrate and acclimatized in
7 an environmental-controlled greenhouse. The height and the weight of shoots
8 were measured again at 4 and 8 weeks after germination.
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19 2.2 Water stress assay, gas exchange measurements and chlorophyll 20 fluorescence analysis 21

22 Three week-old control (non-transformed) and transgenic plants (T0
23 plants) grown in a greenhouse were deprived from water during 3 days. Control
24 plants were irrigated with 50 ml of water every day during the three days
25 duration of the experiment (Faize et al. 2011).
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34 Photosynthetic capacity of leaves was estimated from the analysis of A-
35 C_i response curves. Measurements were made using an open gas exchange
36 system (Li6400, Li-Cor Inc., Nebraska, USA) with an integrated leaf chamber
37 fluorometer (LI-6400-40, Li-Cor Inc., Nebraska, USA). The curves were
38 performed under saturating irradiance (1800 $\mu\text{mol m}^{-2} \text{s}^{-1}$) and under constant
39 leaf temperature (25°C) by controlling the CO₂ concentration of inlet air in 11
40 steps from 50 to 1400 $\mu\text{mol mol}^{-1}$. Diffusion leaks when performing the curves
41 were taking into account by applying the manufacturer's equation to determine
42 the diffusion coefficient. Six leaves per line and water treatment were
43 measured. Photosynthetic parameters maximum rate of carboxylation by
44 ribulose 1,5-biphosphate carboxylase/oxygenase (Rubisco) (V_{cmax}) and the
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1 maximum rate of electron transport (J_{\max}) were determined according to the
2 Farquhar model of leaf photosynthesis (Farquhar *et al.*,1980). The temperature
3 dependencies of photosynthetic parameters were calculated according to
4 Bernacchi *et al.* (2002) modified taking into account the effect of mesophyll
5 conductance (g_m). A-Ci curves were fitted with a non-rectangular hyperbola
6 version of the biochemical model of leaf photosynthesis following Ethier and
7 Livingston (2004). The Ci cut-off point was determined based on the method
8 proposed by Ethier *et al.* (2006). From this analysis $V_{c\max}$, J_{\max} and g_m were
9 determined.
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11 The fluorescence of chlorophyll was measured with a chlorophyll fluorometer
12 (IMAGIM-PAM M-series, Heinz Walz, Effeltrich, Germany) in detached leaves
13 from well-irrigated and water stressed tobacco lines after 3 days. After dark-
14 incubation of plants (15 min), the minimum and the maximal fluorescence yields
15 were monitored. Kinetic analyses were carried out with actinic light ($81 \mu\text{mol}$
16 $\text{quanta m}^{-2} \text{s}^{-1}$ PAR) and repeated pulses of saturating light at $2700 \mu\text{mol}$
17 $\text{quanta m}^{-2} \text{s}^{-1}$ PAR for 0.8 s at intervals of 20 s. The effective PSII quantum
18 yield ($Y(II)$), the non-photochemical quenching (NPQ) and the coefficients of
19 non-photochemical quenching (qN), and the photochemical quenching (qP)
20 were analyzed.
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22 2.3 Mineral content

23 Mineral content was analyzed on the leaves of non-transformed and
24 transgenic tobacco T0 plants grown in normal conditions or exposed to 3 days
25 mild water stress. Leaves were washed with distilled water, dried at $65 \text{ }^\circ\text{C}$,
26 ground and stored at room temperature. Inorganic solute analysis was
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1 performed by the Ionic Services at CEBAS-CSIC (Murcia, Spain). Briefly,
2 samples were digested using a high-performance microwave reaction
3 (Ultraclave; Milestone, Shelton, CT, USA) with HNO₃:H₂O₂ (4:1, v/v) and then
4 used for macro- and micro-nutrient determination with inductively coupled
5 plasma-optical emission spectrometry (ICP-OES), in a Thermo ICP-ICAP 6500
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2.4 Statistical analysis

The effects of the overexpression of *cytsod* and/or *cytapx* on leaf mineral nutrition and gas-exchange parameters measured in non-transformed and transgenic lines under well-irrigated and deficit irrigation conditions were tested by a two-way ANOVA, whereas those related to seedling growth parameters in T1 plants were tested by one-way ANOVA. Within each irrigation treatment, lines were compared with non-transformed controls by a Dunnett's test.

3 Results

3.1 Effect of *cytsod* and *cytapx* on maximum rate of carboxylation by Rubisco (V_{cmax}) and maximum rate of electron transfer (J_{max})

The effect of *cytsod* and *cytapx* on V_{cmax} and J_{max} was investigated in plants well irrigated and in plants subjected to deficit irrigation (Table 1). V_{cmax} data were affected by the "Line" and "Irrigation Treatment" factors and an interaction between both factors was observed. However, J_{max} and the ratio $J_{\text{max}}/V_{\text{cmax}}$ were affected only by the "Irrigation Treatment" factor.

1 Under well irrigated conditions, line 39 displayed a significantly higher
2 V_{cmax} than the non-transformed control, but the transgenic lines 17 and 51
3 presented a significant lower V_{cmax} value than non-transformed line. Line 39
4 also exhibited the highest J_{max} values (Table 1). However, under deficit
5 irrigation conditions, V_{cmax} and J_{max} decreased in all cases and values were
6 quite similar in all tobacco lines. Finally, the ratio J_{max}/V_{cmax} was similar in all
7 lines both under well irrigated and under deficit irrigation conditions although **an**
8 **increase** in non-transformed plants and in line 39 occurred (Table 1).
9

10 The images of chlorophyll fluorescence parameters showed that under well
11 irrigated conditions lines 39 and 51 displayed the highest qP and Y(II) values,
12 whereas non-transformed plants showed the highest values for the non-
13 photochemical quenching parameters (qN and NPQ) (Fig.1). Under deficit
14 irrigation conditions a decrease in the photochemical quenching parameters [qP
15 and Y(II)] as well as the qN parameter was observed in non-transformed plants
16 (Fig.1). A decrease in qP also occurred in line 51 that was accompanied by an
17 increase in the NPQ parameter, related to the safe dissipation of the excess
18 light energy. However, under deficit irrigation conditions, line 17 did not change
19 the qP parameter and even an increase in the non-photochemical parameters
20 (qN and NPQ) was observed. Finally, in line 39 **deficit irrigation** also decreased
21 qP, but ~~unchanged Y(II) and NPQ values were observed~~ (Fig. 1).
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51 3.2 Effect of *cytsod* and *cytapx* and water stress on mineral macro- and micro- 52 elements 53

54 Leaf mineral content (macro and micro-elements) was determined in both
55 non-transformed tobacco lines and transformed lines under deficit of irrigation
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1 and under well irrigated conditions. The ANOVA analysis revealed that the data
2 for all the macro-nutrients, except Ca, were affected by the "Line" studied.
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4 However, the "Irrigation Treatment" factor had a significant effect only in Na and
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6 Mg contents, whereas a significant interaction was observed for the C, Na, Ca
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8 and Mg levels (Table 2). Regarding the micro-nutrients, "Line" and "Irrigation
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10 Treatments" factors, as well as the interaction between them, had a significant
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12 effect only on Mn contents. All the studied micro-nutrients were affected by the
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14 "Line" used. An interaction "Line*Irrigation Treatments" was also observed for
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16 Zn contents (Table 3). Under well irrigated conditions all the transformed lines
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18 showed slightly lower C contents than non-transformed plants (Table 2). Line 39
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20 had significantly higher Na and B contents than non-transformed, whereas line
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22 51 showed higher Mg, B, Mn and Zn levels than the non-transformed plants
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24 (Tables 2 and 3). Under deficit irrigation conditions the most important changes
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26 were observed in line 51 that presented the highest Na, B and Mn contents,
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28 whereas line 17 also showed higher Mn levels than non-transformed plants
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30 (Tables 2 and 3).

3.3 Effect of *cytsod* and *cytapx* on seed germination under various conditions

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42 The effect of ectopic expression of *cytsod* and *cytapx* on seed
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44 germination was investigated in new and aged seeds as well as on seeds
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46 soaked in 100 mM NaCl (Fig. 2). For this experiment, we used seeds produced
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48 by self-pollinating the T0 lines and therefore around 6% of the seeds were non
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50 transgenic (corresponding to a segregation 1:16 of two independently inherited
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52 copies of the *nptII* gene).
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When new seeds were sown in the presence of 100 mM of NaCl germination rate was severely affected in the control (only 2% of seeds germinated after 10 days, Fig. 2A). The percentage of germination was also affected in the line 39 (around 20% of seeds germinated after 10 d). This line showed significant differences in the germination rate with the non-transformed control at 5 days. However, germination rate was less affected in lines 17 and 51 (T50 at 5d and 7d, respectively), reaching a germination rate of about 67% for line 17, and about 58% for line 51 (Fig 2A).

Germination rate was also determined in aged seeds that were stored for 4 years at 8°C (Fig. 2B). Germination was severely affected in the non-transformed seeds (less than 20% of seeds germinated after 10 days), while it was barely affected in the line 39 (T50 of 3.5 d) and not affected in the lines 17 and 51 (T50 of 2 d) (Fig 2B). According to Dunnett's test the three transgenic lines exhibited significantly higher germination ability than the non-transformed control ($P < 0.05$).

3.4 Effect of *cytsod* and *cytapx* on plant vigor and biomass

Transgenic lines (T1 plants) exhibited elevated vigor, showing significantly higher shoot and root length than the non-transformed plants (Fig. 3, Table 4). After 2 weeks, root growth was significantly higher in transgenic tobacco lines than in non-transformed plants. Root length was 2.7-fold and 1.8-fold higher in lines 17 and 39, respectively, whereas in line 51 the effect was even more evident, and root reached a length 4-fold higher than non-transformed plants (Table 4).

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When compared to the non-transformed plants, shoot length was significantly higher in the transgenic lines, being 1.7-fold in line 17 and about 2-fold in lines 39 and 51, after 2 weeks of germination. Four weeks after germination all the transgenic lines showed higher vigor than the non-transformed plants, although values were only statistically significant for lines 17 and 51. Two month after germination, shoot length was about 2.5-fold higher in lines 17 and 39 whereas line 51 reached a shoot length 4.3-fold higher than to non-transformed plants (Table 4).

The transgenic lines showed also higher biomass when compared to the non-transformed plants at 2 weeks after germination, being 2.4-fold higher in the line 17, 1.6-fold in the line 39 and about 4-fold in the line 51 when compared with non-transformed plants. However, differences were significant only for lines 17 and 51 (Table 4).

4 Discussion

We generated transgenic tobacco transformed with *cytsod* (line 17), *cytapx* (line 51) or containing both transgenes (line 39). These transgenic lines showed enhanced tolerance to mild water stress that correlated with higher water use efficiency and better photosynthesis rates, as well as with increased antioxidant capacity in both soluble and chloroplast fractions (Faize et al. 2011). In this work we showed that these transgenic lines exhibited modified photosynthetic capacity, enhanced mineral content and seed germination as well as plant growth.

4.1 Photosynthetic capacity

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2 A direct effect of water stress is disruption of photosynthesis. The
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4 maximum Rubisco activity (V_{cmax}) and electron transport capacity (J_{max}) are
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6 the key parameters determining photosynthetic capacity (Dickson and
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8 Tomlinson, 1996). V_{cmax} is a measurement of the process by which Rubisco
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10 catalyzes the reaction of ribulose 1, 5-biphosphate (RuBP) with CO₂ to produce
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12 the carbon compounds that become triose phosphates, whereas J_{max} reflects
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14 electron transport through the thylakoid membrane, which is critical to produce
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16 NADPH and ATP, and then provide the metabolic energy necessary to produce
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18 triose phosphates. In this study we showed that under well-watered conditions,
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20 lines 39 and 51 exhibited the highest J_{max} values that correlated with high net
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22 photosynthesis (Faize et al. 2011) and the qP parameter. Under deficit irrigation
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24 conditions, decreases in V_{cmax} and J_{max} were observed in all cases that
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26 correlated with decreases in qP and Y(II) and increases in NPQ in non-
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28 transformed plants and in line 51. However, lines 17 and 39 maintained Y(II)
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30 and also increased NPQ as a safe mechanism of excess energy dissipation.
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32 Some authors, point out that the reduction in V_{cmax} can be due to an
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34 inactivation or loss of Rubisco, reducing the carboxylation efficiency, while the
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36 reduction in J_{max} seems to be associated with a diminution of sedoheptulose-1,
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38 7-bisphosphatase, a key regulatory enzyme in the Calvin cycle (Allen et al.
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40 1997; 2000; Nogués and Baker 2000; Ölcer et al 2001). In fact, the decrease in
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42 CO₂ assimilation would reduce the demand for ATP and NADPH, resulting in
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44 decreased electron transport (Zhou et al. 2004). The ratio J_{max}/V_{cmax} can be
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46 considered as a parameter indicating light absorbed/light used for CO₂ fixation.
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48 For this reason, values close to 1 could indicate a balanced photosynthetic
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1 process, as observed in line 39, or even in non-transformed plants under well
2 irrigated conditions. Lines 17 and 51 (harboring only one transgene) showed
3 constitutive high J_{max}/V_{cmax} values. However, values greater than 1, as
4 occurred under deficit irrigation conditions in non-transformed plants and line
5 39, could indicate an excess of light energy that can lead to the photoinhibition
6 of photosynthesis as well as ROS over-generation. In fact, these tobacco lines
7 suffered a higher decrease in photosynthetic rate (A_N) (near 50% decrease)
8 than lines 17 and 51 (about 25% decrease) (Faize et al. 2011), maintaining the
9 J_{max}/V_{cmax} values even under stress conditions. In addition, this response
10 correlated with higher chloroplast and cytosolic APX and SOD levels (Faize et
11 al. 2011), enzymes involved in the water-water cycle, which is involved in
12 dissipation of the excess of excitation energy under environmental stress
13 conditions (Asada 1999).
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31 32 33 34 4.2 Leaf mineral nutrition and plant growth

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36 Micro-elements such as Fe, Cu, Mn and Zn are cofactors of different
37 metalloenzymes, including APX, catalase, peroxidases (POXs) and SOD
38 isoenzymes (Fe-, Mn- and CuZn-SOD), along with their role in other important
39 processes including protein synthesis, enzyme activation or carbohydrate
40 metabolism (Marschner 1997). The content in these micro-elements was
41 somehow higher in transgenic lines than in non-transformed plants. This could
42 be related with the enhanced activity of those metalloenzymes described
43 previously (Faize et al. 2011). Moreover, line 51 presented higher B content
44 under well irrigated and deficit irrigation conditions that was parallel with a better
45 membrane protection (Faize et al. 2011). Boron is an essential micronutrient for
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1 plant growth and development, and it is involved in a number of metabolic
2 pathways and functions such as cell wall synthesis and structure lignification,
3 carbohydrate metabolism, phenol metabolism and plasma membrane integrity
4 (Marschner 1997).
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9 Several factors may contribute to the changes in element contents of the
10 leaves. A large size of the root system will help to access nutrient with limited
11 diffusion such as phosphates (Marschner 1997). Increased leaf mineral nutrient
12 may help to maintain efficient photosynthesis and thus biomass. A correlation
13 between vigor and tolerance to water stress has been reported in several plants
14 such as *Arabidopsis* (Narang et al. 2000) and rice (Price et al. 2002). Under our
15 experimental conditions, overexpression of *cytsod* and/or *cytapx* genes
16 stimulated the growth of T1 tobacco plants. This was especially noticeable in
17 line 51, which exhibited better growth parameters than the non-transformed
18 control. Under normal conditions, as well as under drought stress, line 51
19 exhibited the highest maximum net photosynthesis (A_n) that showed a good
20 relationship with leaf mass area (Faize et al. 2011). In addition, the elevated qP
21 and $Y(II)$ values observed for this line are indicative of its better photosynthetic
22 capacity. Moreover, line 51 showed a better membrane protection as observed
23 by its lower TBARS content and electrolyte leakage values (Faize et al. 2011).
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48 4.3 Seed Physiology 49

50 Our transgenic tobacco plants exhibited a tight control of ROS production
51 via an enhanced antioxidant capacity (Faize et al. 2011). ROS can act as
52 signaling molecules regulating plant growth and development (Gechev et al.
53 2006), being also involved in seed germination and seedling growth (Kranmer et
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al. 2010; Barba-Espín et al. 2010). Increased growth by over-expression of antioxidants enzymes has also been reported in transgenic tobacco expressing pepper ascorbate peroxidase-like gene (Sarowar et al. 2005).

The reactivation of metabolism following seed imbibition may provide an important source of ROS (Bailly 2004; Kraner et al. 2010). For this reason, anti-oxidative mechanisms have been regarded as being of particular importance for the success of germination, and most of them increase during germination (De Gara et al. 1997; Tommasi et al. 2001; Bailly 2004; Barba-Espín et al. 2010). Uncontrolled ROS accumulation during germination may lead to seed deterioration. For this reason ROS accumulation must be tightly regulated by the scavenging mechanisms during seed germination (Bailly 2004). These deleterious effects of ROS, with the concomitant decrease in the antioxidative defenses, are responsible for decreased structural integrity and increased seed mortality (Priestley et al. 1985; Simrnoff 1993). Therefore, reinforcement of antioxidative system can lead to improved seed germination and plant vigor. In this sense, our results showed that overexpression of *cytapx* or *cytsod* increased seed germination, vigor and plant biomass under non-stress conditions in T1 plants.

Salt stress can affect different physiological processes, including seed germination (Darra et al. 1973; Heikal et al. 1982). Salinity is detrimental to both seed viability and vigor during germination (Unger 1978). In salt-sensitive species, germination under saline conditions produced osmotic potential increases which can inhibit the imbibition process by restricting water uptake (Bradford 1990). In the present work, we showed that salt stress drastically reduced the rate of germination in non-transformed tobacco being transgenic

lines less affected. Surprisingly, line 39, overexpressing both transgenes, exhibited an intermediate phenotype. This differential behavior could rely on the modulation of the activity of ROS scavenging enzymes (Faize et al. 2011). It has been extensively reported that salt stress induced an oxidative stress in plants (Hernández et al. 2001). In this way, the overexpression of antioxidants enzymes (APX and/or Cu,Zn-SOD) can lead to an optimal control of ROS generated during the germination process under salinity conditions. The addition of antioxidants such as reduced ascorbate or glutathione improved the germination rate of *Arabidopsis* seeds under NaCl stress (Borsani et al. 2001). Probably, the overexpression of *cytsod* or *cytapx* can minimize the salt-induced oxidative stress during the germination of tobacco seeds, improving their germination rates. Moreover, different works reported that ROS, when tightly regulated, can be beneficial for the germination process (Bailly et al. 2008; Kranner et al. 2010; Barba-Espín et al. 2010; 2011; Diaz-Vivancos et al. 2013). In previous work, we reported that the imbibition of pea seeds with low H₂O₂ levels increased the germination rate and the early seedling growth, and this response was correlated with the induction of cytosolic and stromatic *apx* transcripts and their corresponding activity as well as with the increase of other antioxidant enzymes such as monodehydroascorbate reductase (MDHAR) and POX (Barba-Espín et al. 2010). Improvement of seed germination using tylokoidal *apx* or plastidial *sod* and *apx* has been already reported in tobacco (Sun et al. 2010; Lee et al. 2010). However, this is the first report of the involvement of cytosolic ROS-scavenging enzymes during germination.

Seed germination is reported to be either suppressed or slowed down during aging (McDonald 1999). In our conditions, seed storage for 4 years

1 severely affected germination in non-transformed tobacco, while it had no
2 detrimental effect on germination of transgenic seeds, probably due to the lack
3 of detrimental ROS accumulation during storage. Viability losses during storage
4 is a result of ROS accumulation as well as a decrease in antioxidant
5 mechanisms, which ultimately lead to oxidative damage to essential
6 macromolecules during seed imbibition (Bailly et al. 2008; Rajjou et al. 2008).
7 Therefore, the regulation of ROS generation and antioxidant defenses is of vital
8 importance to maintain seed viability. In our work, aged seeds from transformed
9 lines showed no loss of viability after 10 days post-imbibition, contrary to the
10 control seeds, which showed a very low germination rate, indicating serious
11 seed deterioration during storage. Our results are in agreement with those of
12 Lee et al. (2010) who showed that overexpression of SOD and APX in tobacco
13 plastids resulted in enhanced seed viability of aged seeds. Their transgenic
14 lines exhibited low electrolyte leakage and maintained their membrane integrity
15 during imbibition, due to higher antioxidant ability, which results in a lower
16 accumulation of ROS. Our results demonstrate that overexpression of *cytsod*
17 and *cytapx* in the cytosol can also increase seed longevity and germination
18 rates under unfavorable environments.

45 **5 Conclusions**

46 Taken together, these results suggest that cytosolic antioxidants
47 mechanisms can be useful tools to improve several physiological processes
48 such as seed germination, nutrient uptake, plant growth and a better
49 photosynthetic response under abiotic stress conditions.
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References

- 21
22
23
24 Allen DJ, McKee IF, Farage PK, Baker NR (1997) Analysis of the limitation to CO₂
25
26 assimilation on exposure of leaves of two *Brassica napus* cultivars to UV-B.
27
28 Plant Cell Environ 20: 633–640.
29
30
31 Alscher RG, Donahue JL, Cramer CL (1997) Reactive oxygen species and
32
33 antioxidants: relationships in green cells. *Physiol Plant* 100: 224-223.
34
35
36 Asada K (1999) The water–water cycle in chloroplasts: scavenging of active oxygens
37
38 and dissipation of excess photons. *Ann Rev Plant Physiol Plant Mol Biol* 50:
39
40 601-639.
41
42
43 Bailly C, Bogatek-Leszczynska R, Côme D, Corbineau F (2002) Changes in activities
44
45 of antioxidant enzymes and lipoxygenase during growth of sunflower
46
47 seedlings from seeds of different vigour. *Seed Sci Res* 12:47-55,
48
49
50
51 Bailly C (2004) Active oxygen species and antioxidants in seed biology. *Seed Sci*
52
53 *Res* 14: 93-107.
54
55
56
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60
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62
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49
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51
52
53
54
55
56
57
58
59
60
61
62
63
64
65
- Bailly C, El-Maarouf-Bouteau H, Corbineau F (2008) From intracellular signaling networks to cell death: the dual role of reactive oxygen species in seed physiology. *Compt. Rend. Biol.* 331: 806-814.
- Barba-Espin G, Diaz-Vivancos P, Clemente-Moreno MJ, Albacete A, Faize L, Faize M, Pérez-Alfocea F, Hernández JA (2010) Interaction between hydrogen peroxide and plant hormones during germination and the early growth of pea seedlings. *Plant Cell Environ* 33: 981-994.
- Barba-Espin G, Diaz-Vivancos P, Job D, Belghazi M, Job C, Hernández JA (2011) Understanding the role of H₂O₂ during pea seed germination: a combined proteomic and hormone profiling approach. *Plant Cell Environ* 34: 1907-1919.
- Bartoli CG, Gomez F, Martinez DE, Guiamet JJ (2004) Mitochondria are the main target for oxidative damage in leaves of wheat (*Triticum aestivum* L.). *J Exp Bot* 55: 1663–1669.
- Bernacchi CJ, Portis AR, Nakano H, von Caemmerer S, Long SP (2002) Temperature response of mesophyll conductance. Implications for the determination of Rubisco enzyme kinetics and for limitations to photosynthesis *in vivo*. *Plant Physiol* 130: 1992-1998.
- Borsani O, Valpuesta V, Botella MA (2001) Evidence for a role of salicylic acid in the oxidative damage generated by NaCl and osmotic stress in *Arabidopsis* seedlings. *Plant Physiol* 126: 1024-1030.
- Brandford KJ (1990) A water relations analysis of seed germination rates. *Plant Physiol* 94: 840-849.
- Cakmak I, Strbac D, Marschner H (1993) Activities of hydrogen peroxide-scavenging enzymes in germinating wheat seeds. *J Exp Bot* 44: 127-132.

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60
61
62
63
64
65
- Caliskan M, Cuming AC (1998) Spatial specificity of H₂O₂-generating oxalate oxidase gene expression during wheat embryo germination. *Plant J* 15: 165-171,
- Darra BL, Seth SP, Singh H, Mendriatta RS (1973) Effect of hormone directed presoaking on emergence and growth of osmotically stressed wheat (*Triticum sativum* L) seeds. *Agron J* 65: 292-295,
- De Gara L, de Pinto MC, Arrigoni O (1997) Ascorbate synthesis and ascorbate peroxidase activity during the early stage of wheat germination. *Physiol Plant* 100: 894–900,
- Diaz-Vivancos P, Rubio M, Mesonero V, Periago PM, Ros Barceló A, Martínez-Gómez P, Hernández JA (2006) The apoplastic antioxidant system in *Prunus*: Response to plum pox virus. *J Exp Bot* 57: 3813-3824.
- Diaz-Vivancos P, Barba-Espín G, Hernández JA (2013) Elucidating hormonal/ROS networks during seed germination: insights and perspectives. *Plant Cell Rep* 32: 1491-1502.
- Dickson R, Tomlinson P (1996) Oak growth, development and carbon metabolism in response to water stress. *Ann For Sci* 53: 181-196.
- Ethier GJ, Livingston NJ (2004) On the need to incorporate sensitivity to CO₂ transfer conductance into the Farquhar–von Caemmerer–Berry leaf photosynthesis model. *Plan Cell Environ* 27: 137–153.
- Ethier GJ, Livingston NJ, Harrison DL, Black TA, Moran JA (2006) **Low stomatal and internal conductance to CO₂ versus Rubisco** deactivation as determinants of the photosynthetic decline of ageing evergreen leaves. *Plant Cell Environ* 29: 168-2184.

1 Faize M, Burgos L, Faize L, Piqueras A, Nicola, E, Barba-Espin G, Clemente-Moreno
2 MJ, Alcobendas R, Artlip T, Hernandez JA (2011) Involvement of cytosolic
3 ascorbate peroxidase and Cu/Zn-superoxide dismutase for improved
4 tolerance against drought stress. J Exp Bot 62: 2599-2613.
5
6
7

8
9 Farquhar GD, von Caemmerer S, Berry JA (1980) A biochemical-model of
10 photosynthetic CO₂ assimilation in leaves of C-3 species. Planta 149: 78-90.
11
12

13 Flexas J, Bota J, Escalona JM, Sampol B, Medrano H (2002) Effects of drought on
14 photosynthesis in grapevines under field conditions: an evaluation of stomatal
15 and mesophyll limitations. Funct Plant Biol 29: 461-471.
16
17
18

19 Fridovich I (1975) Superoxide dismutases. Ann Rev Biochem 44: 147-149.
20
21

22 **Gaxiola RA, Li J, Undurraga S, Dang LM, Allen GJ, Alper SL, Fink GR (2001)**
23
24 Drought- and salt-tolerant plants result from overexpression of the AVP1 H1-
25 pump. Proc Natl Acad Sci USA 98: 11444-11449.
26
27
28

29 Gechev TS, Van Breusegem F, Stone JM, Denev I, Laloi C (2006) Reactive oxygen
30 species as signals that modulate plant stress responses and programmed cell
31 death. BioEssays 28: 1091-1101.
32
33

34 **Gidrol X, Lin W, Degousse N, Yip SF, Kush A (1994) Accumulation of reactive**
35 **oxygen species and oxidation of cytokinin in germinating soybean seeds. Eur**
36 **J Biochem 224: 21-28.**
37
38

39 **Gu J, Yin X, Stomph TJ, Wang H, Struik PC (2012) Physiological basis of genetic**
40 **variation in leaf photosynthesis among rice (*Oryza sativa* L.) introgression**
41 **lines under drought and well-watered conditions. J Exp Bot 63: 5137-5153.**
42
43
44

45 Heikal MM, Shaddad MA, Ahmed MM (1982) Effect of water stress and gibberellic
46 acid on germination of flax, sesame and onion seeds. Biol Plant 24: 124-129.
47
48
49
50
51
52
53
54
55
56
57
58
59
60
61
62
63
64
65

1 Hendry GAF (1993) Oxygen, free radical processes and seed longevity. Seed Sci
2 Res 3: 141-153.
3

4 Hernández JA, Corpas FJ, Gómez M, del Río LA, Sevilla F (1993) Salt-induced
5 oxidative stress mediated by activated oxygen species in pea leaf
6 mitochondria. Physiol Plant 89: 103-110.
7
8
9

10 Hernández JA, Ferrer MA, Jiménez A, Ros-Barceló A, Sevilla F (2001) Antioxidant
11 systems and $O_2\cdot^-/H_2O_2$ production in the apoplast of *Pisum sativum* L. leaves:
12 its relation with NaCl-induced necrotic lesions in minor veins. Plant Physiol
13 127: 817-831.
14
15
16
17
18
19
20

21 Jiménez A, Hernández JA, del Río LA, Sevilla F (1997) Evidence for the presence
22 of the ascorbate-glutathione cycle in mitochondria and peroxisomes of pea
23 (*Pisum sativum* L.) leaves. Plant Physiol 114: 275-284.
24
25
26
27
28

29 Kranner I, Roach T, Beckett RP, Whitaker C, Minibayeva FV (2010) Extracellular
30 production of reactive oxygen species during seed germination and early
31 growth in *Pisum sativum*. J Plant Physiol 167: 805-811.
32
33
34
35

36 Lee YP, Baek KH, Lee HS, Kwak SS, Bang JW, Kwon SY (2010) Tobacco seeds
37 simultaneously over-expressing Cu/Zn superoxide dismutase and ascorbate
38 peroxidase display enhanced seed longevity and germination rates under
39 stress conditions. J Exp Bot 61: 2499-2506.
40
41
42
43
44
45

46 Leymarie J, Vitkauskaite V, Hoang H, Gendreau E, Chazoule V, Meimoun F,
47 Corbineau F, El-Maarouf-Bouteau H, Bailly C (2012) Role of reactive oxygen
48 species in the regulation of Arabidopsis seed dormancy. Plant Cell Physiol 53:
49 96-106.
50
51
52
53
54

55 Marschner H (1997) Mineral nutrient of higher plants. London Academic Press Inc
56 Second Edition, London, pp 313-396.
57
58
59
60
61
62
63
64
65

1 McDonald MB (1999) Seed deterioration: physiology, repair and assessment. Seed
2 Sci Technol 27: 177-237.
3

4 Mittova V, Tal M, Volokita M, Guy M (2003). Up-regulation of the leaf mitochondrial
5 and peroxisomal antioxidative systems in response to salt-induced oxidative
6 stress in the wild salt-tolerant tomato species *Lycopersicon pennellii*. Plant
7 Cell Environment 26: 845-856.
8
9

10 Morohashi Y (2002) Peroxidase activity develops in the micropylar endosperm of
11 tomato seeds prior to radicle protrusion. J Exp Bot 53:1643-1650,
12
13

14 Miller G, Suzuki N, Ciftici-Yilmazi S, Mittler R (2010) Reactive oxygen species
15 homeostasis and signaling during drought and salinity stresses. Plant Cell
16 Environment 33: 453-467.
17
18

19 Narang RA, Bruene A, Altmann T (2000) Analysis of phosphate acquisition efficiency
20 in different Arabidopsis accessions. Plant Physiol 124: 1786-1799.
21
22

23 Noctor G, Foyer C (1998) Ascorbate and glutathione: keeping active oxygen under
24 control. Ann Rev Plant Physiol Plant Mol Biol 49: 249-279.
25
26

27 Nogués S, Baker NR (2000) Effects of drought on photosynthesis in Mediterranean
28 plants grown under enhanced UV-B radiation. J Exp Bot 51:1309-1317.
29
30

31 Ölcer H, Llyod JC, Raines CA (2001) Photosynthetic capacity is differentially affected
32 by reduction in sedoheptulose, 1-7 bis phosphatase activity during leaf
33 development in transgenic tobacco plants. Plant Physiol 125: 982-989.
34
35

36 Oracz K, El-Maarouf-Bouteau H, Kranner I, Bogatek R, Corbineau F, Bailly C (2009)
37 The mechanisms involved in seed dormancy alleviation by hydrogen cyanide
38 unravel the role of reactive oxygen species as key factors of cellular signaling
39 during germination. Plant Physiol 150: 494-505.
40
41
42
43
44
45
46
47
48
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45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60
61
62
63
64
65
- Parish DJ, Leopold AC, Hanna MA (1982) Turgor changes with accelerated ageing of soybeans. *Crop Sci* 22: 666-669.
- Perl-Treves R, Perl A (2002) Oxidative stress: An introduction. In: Inzé D, Van Montagu M (eds) *Oxidative Stress in Plants*. Taylor and Francis, London, pp 1-32.
- Price AH, Cairns JE, Horton P, Jones HG, Griffiths H (2002) Linking drought-resistance mechanisms to drought avoidance in upland rice using a QTL approach: Progress and new opportunities to integrate stomatal and mesophyll responses. *J Exp Bot* 53: 989-1004.
- Priestley DA, Warner BG, Leopold AC, McBride MB (1985) Organic free radical levels in seeds and pollen: the effects of hydration and ageing. *Physiol Plant* 70: 88-94.
- Puntarulo S, Galleano M, Sanchez RA, Boveris A (1991) Superoxide anion and hydrogen peroxide metabolism in soybean embryonic axes during germination. *Biochim Biophys Acta* 1074:277-283,
- Rajjou L, Lovigny Y, Groot SPC, Belghazi M, Job C, Job D (2008) Proteome-wide characterization of seed aging in *Arabidopsis*: A comparison between artificial and natural aging protocols. *Plant Physiol* 148: 620-641.
- Sarowar S, Kim EN, Kim YJ, Ok SH, Kim KD, Hwang BK, Shin JS (2005) Overexpression of a pepper ascorbate peroxidase-like 1 gene in tobacco plants enhances tolerance to oxidative stress and pathogens. *Plant Sci* 169: 55-63.
- Schopfer P, Plachy C, Frahy G (2001) Release of reactive oxygen intermediates (superoxide radicals, hydrogen peroxide, and hydroxyl radicals) and

peroxidase in germinating radish seeds controlled by light, gibberellin, and abscisic acid. *Plant Physiol* 125: 1591-1602,

Smirnoff N (1993) The role of active oxygen in the response of plants to water deficit and desiccation. *New Phytol* 125: 27-58.

Sun WH, Duan M, Shu DF, Yang S, Meng QW (2010) Over-expression of *StAPX* in tobacco improves seed germination and increase early seedling tolerance to salinity and osmotic stresses. *Plant Cell Rep* 29: 917-926.

Tommasi F, Paciolla C, de Pinto MC, De Gara L (2001) A comparative study of glutathione and ascorbate metabolism during germination of *Pinus pinea* L. seeds. *J Exp Bot* 52: 1647–1654.

Ungar IA (1978) Halophyte seed germination. *Bot Rev* 44: 233-264.

Zhou YH, Yu JQ, Huang LF, Nogués S (2004) The relationship between CO₂ assimilation, photosynthetic electron transport and water–water cycle in chill-exposed cucumber leaves under low light and subsequent recovery. *Plant Cell Environ* 27: 1503–1514.

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Figure Captions

Figure 1. Effect of deficit irrigation on chlorophyll fluorescence parameters in leaves of non-transformed (NT) and transformed tobacco lines (17, 39 and 51). Images of the coefficient of photochemical quenching (qP), the effective PSII quantum yield [Y(II)], the coefficient of non-photochemical quenching (qN) and the non-photochemical quenching (NPQ) parameter are shown. I, irrigated; DI, deficit irrigation.

Figure 2. A) Germination percentage from new tobacco seeds imbibed in 100 mM NaCl. B) Germination percentage from aged tobacco seeds. New seeds derived from non-transformed control (NT) and transformed tobacco lines (line 17, line 39 and line 51) were harvested 2 months after anthesis and stocked for 4 years at 8°C. Data are mean and standard errors from 4 independent replicates. 50 seeds were used for each replicate.

Figure 3. Picture showing seedling growth of non-transformed (NT) and transformed tobacco lines (line 17, line39, and line 51) 3 weeks after seed germination. Bar, 1 cm.

Table 1. Maximum rate of carboxylation by ribulose 1-5-bisphosphate carboxylase/oxygenase (Rubisco) ($V_{c,max}$; $\mu\text{mol CO}_2 \text{ m}^{-2} \text{ s}^{-1}$), maximum rate of electron transport (J_{max} , $\mu\text{mol e}^{-1} \text{ m}^{-2} \text{ s}^{-1}$) and $J_{max}:V_{c,max}$ ratio in the different irrigated (I) and deficit irrigation (DI) genotypes lines. Data are the mean \pm SE from 4 replicates.

| <i>Treatment</i> | <i>Line</i> | <i>Vcmax</i> | <i>Jmax</i> | <i>Jmax/Vcmax</i> |
|-----------------------------|-------------|------------------|------------------|-------------------|
| Irrigated | Control | 91.8 \pm 18.2 | 101.5 \pm 21.1 | 1.11 \pm 0.23 |
| | 17 | 71.9 \pm 1.6* | 100.4 \pm 5.1 | 1.40 \pm 0.07 |
| | 39 | 148.4 \pm 8.6* | 140.7 \pm 12.6 | 0.95 \pm 0.12 |
| | 51 | 79.2 \pm 11.6* | 113.06 \pm 7.5 | 1.43 \pm 0.09 |
| Deficit irrigation | | | | |
| | Control | 62.1 \pm 2.2 | 92.5 \pm 6.8 | 1.49 \pm 0.08 |
| | 17 | 55.4 \pm 9.1 | 78.1 \pm 2.1 | 1.41 \pm 0.23 |
| | 39 | 50.2 \pm 2.8 | 82.3 \pm 5.5 | 1.64 \pm 0.15 |
| | 51 | 56.3 \pm 4.4 | 78.1 \pm 7.2 | 1.39 \pm 0.07 |
| ^aF values | | | | |
| <i>Source of variation</i> | | <i>Vcmax</i> | <i>Jmax</i> | <i>Jmax/Vcmax</i> |
| Line (A) | | 5.06** | 1.06 | 1.05 |
| Irrigation Treatment (B) | | 50.04*** | 17.49*** | 9.08** |
| AxB | | 9.00*** | 1.49 | 1.71 |

^aF values from two-way ANOVA for $V_{c,max}$, J_{max} and $J_{max}/V_{c,max}$. F-values significant at 99.9% (***), 99% (**) or 95% (*) levels of probability.

Table 2. Macronutrient contents in leaves from non-transformed and transformed tobacco lines (control, line 17, line39, and line 51) under irrigated (I) and deficit irrigation (DI) conditions (3 days of withholding water). Data are the mean \pm SE from 3 replicates. Asterisks represent significant differences ($P < 0.05$) between each transgenic line and the wild type control, within each irrigation treatment, according to Dunnett's test.

| Treatment | Line | C (%) | N (%) | P (%) | K (%) | Na (%) | Ca (%) | Mg (%) | S (%) |
|-----------|---------|----------------------------------|-----------------|------------------|-----------------|-----------------------------------|-----------------|----------------------------------|------------------|
| I | Control | 41.8 \pm 0.19 | 1.74 \pm 0.14 | 0.308 \pm 0.02 | 3.22 \pm 0.14 | 0.045 \pm 0.01 | 1.65 \pm 0.10 | 0.52 \pm 0.015 | 0.26 \pm 0.014 |
| | Line 17 | 41.1\pm0.23* | 2.06 \pm 0.28 | 0.355 \pm 0.01 | 3.50 \pm 0.48 | 0.047 \pm 0.01 | 1.92 \pm 0.28 | 0.55 \pm 0.011 | 0.28 \pm 0.025 |
| | Line 39 | 41.1\pm0.20* | 2.48 \pm 0.13 | 0.399 \pm 0.04 | 4.20 \pm 0.32 | 0.094\pm0.03* | 1.93 \pm 0.17 | 0.54 \pm 0.01 | 0.32 \pm 0.01 |
| | Line 51 | 41.1\pm0.10* | 2.78 \pm 0.27 | 0.416 \pm 0.04 | 4.17 \pm 0.4 | 0.057 \pm 0.01 | 2.09 \pm 0.21 | 0.62\pm0.03* | 0.35 \pm 0.03 |
| DI | Control | 41.3 \pm 0.19 | 2 \pm 0.41 | 0.338 \pm 0.01 | 3.60 \pm 0.43 | 0.090 \pm 0.01 | 1.85 \pm 0.15 | 0.54 \pm 0.024 | 0.28 \pm 0.032 |
| | Line 17 | 41.8 \pm 0.28 | 1.86 \pm 0.24 | 0.324 \pm 0.04 | 2.96 \pm 0.39 | 0.089 \pm 0.01 | 1.72 \pm 0.14 | 0.48 \pm 0.037 | 0.25 \pm 0.032 |
| | Line 39 | 41.6 \pm 0.21 | 2.84 \pm 0.28 | 0.385 \pm 0.01 | 4.41 \pm 0.23 | 0.078 \pm 0.01 | 1.88 \pm 0.09 | 0.53 \pm 0.007 | 0.32 \pm 0.01 |
| | Line 51 | 40.6 \pm 0.57 | 2.51 \pm 0.07 | 0.434 \pm 0.02 | 4.66 \pm 0.35 | 0.175\pm0.01* | 1.75 \pm 0.24 | 0.58 \pm 0.03 | 0.36 \pm 0.01 |

| Source of variation | ^a F values | | | | | | | | |
|--------------------------|-----------------------|----------|----------|----------|----------|-------|----------|----------|--|
| Line (A) | 6.42** | 18.07*** | 13.67*** | 17.26*** | 13.95*** | 0.76 | 13.78*** | 19.96*** | |
| Irrigation Treatment (B) | 0.11 | 0.11 | 0.02 | 0.89 | 56.0*** | 1.15 | 5.96* | 0.12 | |
| AxB | 5.57** | 2.41 | 1.44 | 2.53 | 20.33*** | 3.43* | 4.42* | 1.10 | |

^aF values from two-way ANOVA for the different macronutrient analysed. F-values significant at 99.9% (***), 99% (**) or 95% (*) levels of probability.

Table 3. Micronutrient contents in leaves from non-transformed and transformed tobacco lines (control, line 17, line39, and line 51) under irrigated (I) and deficit irrigation (DI) conditions (3 days of withholding water). Data are the mean \pm SE from 3 replicates. Asterisks represent significant differences ($P < 0.05$) between each transgenic line and the wild type control, within each irrigation treatment, according to Dunnett's test.

| Treatment | Line | Fe (ppm) | B (ppm) | Cu (ppm) | Mn (ppm) | Zn (ppm) |
|-----------|---------|-------------------|-----------------------------------|-----------------|----------------------------------|-----------------------------------|
| I | Control | 45.08 \pm 5.61 | 28.94 \pm 0.64 | 3.99 \pm 0.71 | 0.52 \pm 0.015 | 21.54 \pm 1.42 |
| | Line 17 | 48.98 \pm 4.72 | 31.52 \pm 2.12 | 4.51 \pm 0.97 | 0.55 \pm 0.011 | 24.88 \pm 2.55 |
| | Line 39 | 57.39 \pm 7.03 | 33.54\pm2.51* | 5.85 \pm 0.2* | 0.54 \pm 0.01 | 23.51 \pm 0.12 |
| | Line 51 | 71.24 \pm 9.02 | 41.9\pm1.03* | 6.6 \pm 0.9* | 0.62\pm0.03* | 30.78\pm2.78* |
| DI | Control | 38.26 \pm 12.45 | 34.44 \pm 2.15 | 4.4 \pm 1.56 | 0.54 \pm 0.024 | 24.87 \pm 1.74 |
| | Line 17 | 45.19 \pm 5.94 | 31.08 \pm 1.18 | 4.78 \pm 1.36 | 0.48\pm0.00* | 23.11 \pm 3.32 |
| | Line 39 | 61.79 \pm 5.94 | 34.11 \pm 1.17 | 5.35 \pm 0.79 | 0.53 \pm 0.007 | 28.35 \pm 3.68 |
| | Line 51 | 58.08 \pm 6.88 | 40.87\pm1.98* | 6.04 \pm 0.16 | 0.58\pm0.03* | 28.23 \pm 0.32 |

| Source of variation | ^a F values | | | | | |
|--------------------------|-----------------------|----------|--------|-----------|--------|--|
| Line (A) | 15.21*** | 44.78*** | 7.84** | 6.05** | 8.62** | |
| Irrigation Treatment (B) | 3.12 | 2.96 | 0.24 | 112.06*** | 1.02 | |
| AxB | 2.54 | 4.49* | 0.72 | 7.54** | 3.68* | |

^aF values from two-way ANOVA for the micronutrient analysed. F-values significant at 99.9% (***), 99% (**) or 95% (*) levels of probability.

Table 4.- Seedling growth parameters at various days after seed imbibition (dai) in non-transformed and in transgenic tobacco lines. Data are means and standard error from 5 to 15 replicates. Asterisks represent significant differences ($P < 0.05$) between each transgenic line and the wild type control, within each irrigation treatment, according to a Dunnett's test.

| Line | Root length (mm) | Shoot length (mm) | | | Shoot fresh weight (mg) |
|-----------------------|---------------------|----------------------|---------|----------|----------------------------|
| | 14 dai | 14 dai | 28 dai | 60 dai | 14 dai |
| Control | 2.9±0.4 | 7.6±1.8 | 71±14 | 78±6 | 308±88 |
| 17 | 7.8±0.9* | 12.7±2.1* | 129±33* | 179±13 | 733±186* |
| 39 | 5.1±0.8* | 15.7±1.8* | 94±10 | 196±42 | 508±51 |
| 51 | 11.6±1.1* | 17.2±2* | 129±29* | 340±123* | 1192±191* |
| ^a F values | | | | | |
| | 96.77*** | 18.09*** | 5.23** | 10.99*** | 27.22*** |

^aF values from one-way ANOVA for the growth parameters analysed. F-values significant at 99.9% (***), 99% (**) or 95% (*) levels of probability.

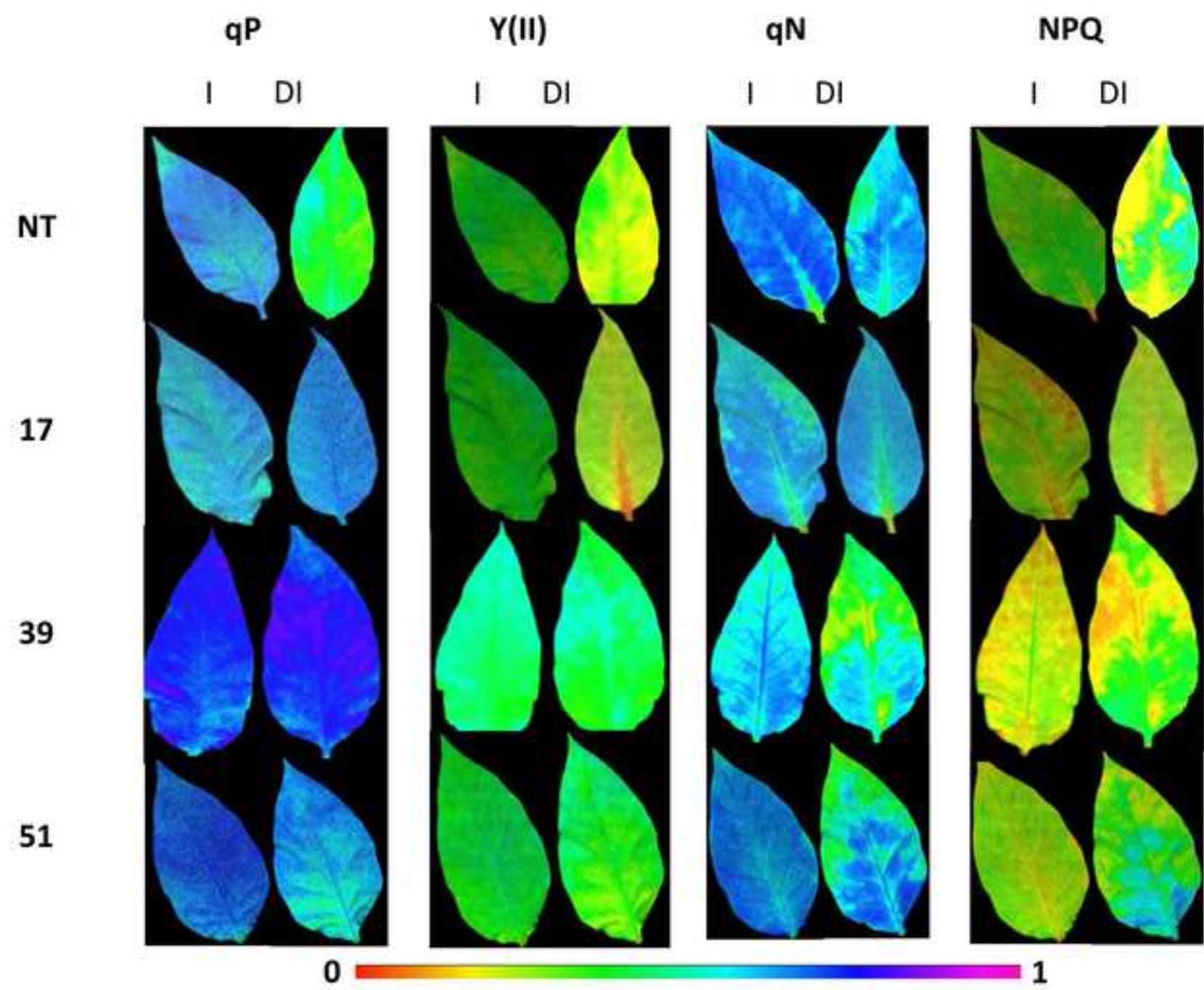


Figure 1.

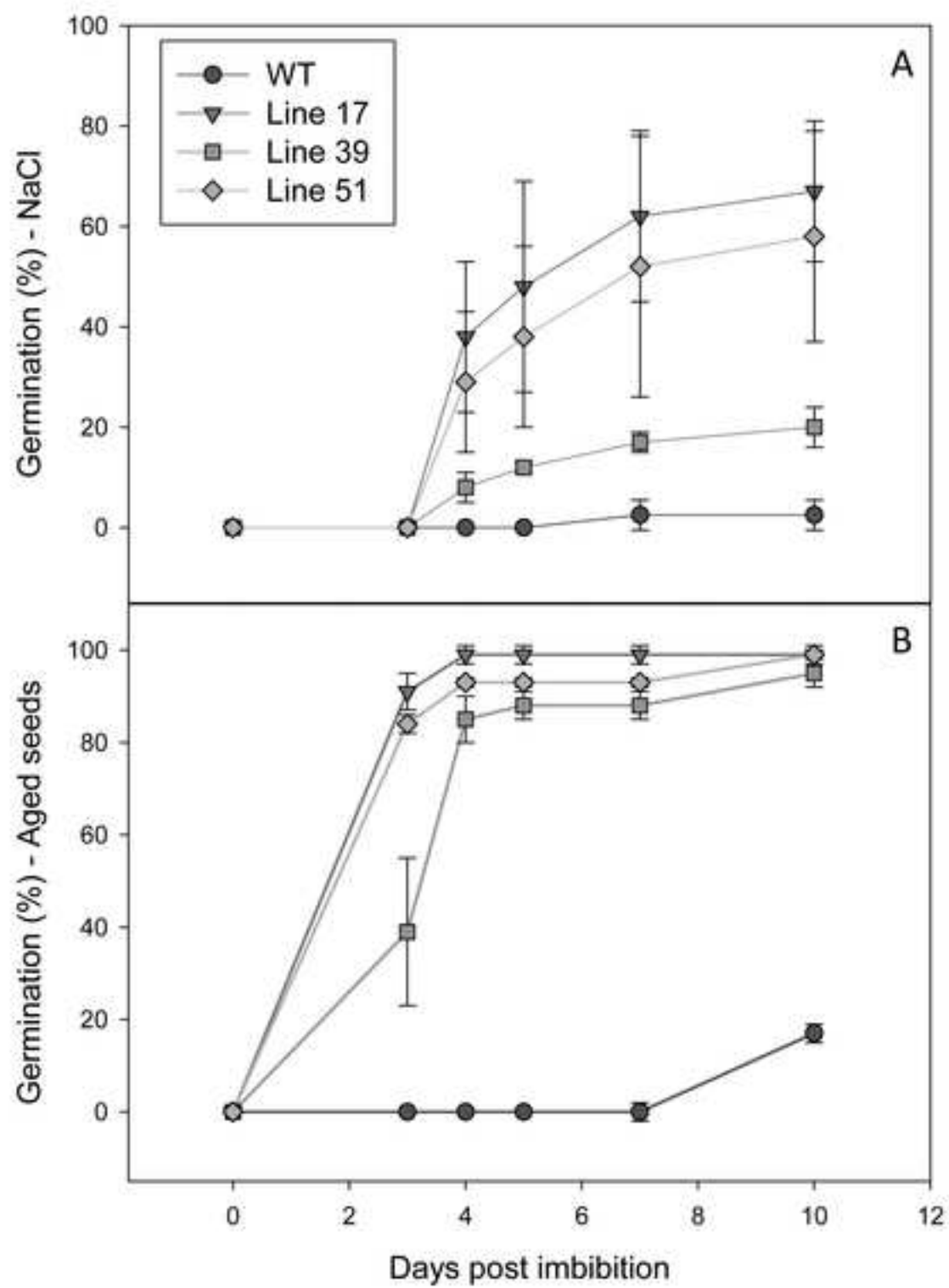


Figure 2.