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Short communication

# Extracellular superoxide production associated with secondary root growth following desiccation of *Pisum sativum* seedlings

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ABSTRACT

The seedling stage is arguably the most vulnerable phase in the plant life cycle, where the young establishing plant is extremely sensitive to environmental stresses such as drought. Here, the production of superoxide ($O_2^{\bullet-}$), a molecule involved in stress signaling, was measured in response to desiccation of *Pisum sativum* L. seedlings. Following desiccation that was sufficient to kill the radicle meristem, viability could be retained by seedlings that grew secondary roots. Upon rehydration, secondary roots formed in a region that had displayed intense extracellular $O_2^{\bullet-}$ production on desiccation. Treating partially desiccated seedlings with hydrogen peroxide ($H_2O_2$) prevented viability loss. In summary, reactive oxygen species (ROS) appear to participate in the signaling required for secondary root formation following desiccation stress of *P. sativum* seedlings.



## Introduction

The transition from seed to seedling coincides with a rapid change in stress tolerance, and especially in desiccation tolerance (Kranner et al., 2010a). The majority of flowering plants produce desiccation tolerant "orthodox" seeds (Dickie et al., 2002), which lose their desiccation tolerance at the later stages of germination (Senaratna and McKersie, 1983; Reisdorph and Koster, 1999) and tolerance can only be regained through sophisticated manipulation within a limited time window (Buitink et al., 2006). Desiccation can increase the production of ROS, which can irreversibly damage macromolecules, but are also implicated in stress signaling and in developmental processes including cell elongation, cell division and differentiation (Kranner and Birtic, 2005; Gapper and Dolan, 2006; Møller et al., 2007; Colville and Kranner, 2011). Following germination, the emerging seedling radicle meristem is particularly sensitive to abiotic stress, predation and damage, but loss of the primary root is not always critical. Partly desiccated seedlings can survive by growing secondary roots (Farrant et al., 2004).

We previously showed that post-germination growth of *Pisum sativum* seedlings is accompanied by a release of extracellular $O_2^{\bullet-}$ from the radicle that coincided with increased activity of extracellular peroxidases (ECPOX) (Kranner et al., 2010b). ECPOX can produce $O_2^{\bullet-}$ in the presence of $H_2O_2$ and reductants (Halliwell, 1978; Roach et al., 2010). $O_2^{\bullet-}$ signaling has been associated with root hair growth (Foreman et al., 2003), cellular organization (Gapper and Dolan, 2006) and root gravitropism (Joo et al., 2001). In addition, growth of adventitious roots requires the presence of $H_2O_2$ (Li et al., 2007; Huang et al., 2011). Catalyzed by superoxide dismutase, $O_2^{\bullet-}$ can be converted to $H_2O_2$, which can reversibly oxidize redox receptors, altering protein structure and function, thus perpetuating the signal (Stone and Yang, 2006). To further investigate ROS signaling in relation to seedling establishment, we studied the production of $O_2^{\bullet-}$ and the effects of $H_2O_2$ treatment on *P. sativum* seedlings in response to non-lethal desiccation such as may occur during transient periods of drought.

## Results and discussion

### *Seedling desiccation, viability and rates of $O_2^{\bullet-}$ production*

After 56 h of seed imbibition, 96% of all seeds had germinated. The resulting seedlings were used for experimentation after the cotyledons and seed coats had been removed to reduce sample size in the analytical procedures. When the remaining seedling axes, hereafter termed "seedlings," were placed over silica gel, water loss immediately started at the radicle tips, and within 6 h spread to the shoots tips (Fig. 1a). Desiccation decreased seedling viability, which was completely lost after 24 h (Fig. 1b).

*Abbreviations:* ECPOX, extracellular peroxidases; FW, fresh weight; $H_2O_2$, hydrogen peroxide; WC, water content; NBT, nitroblue tetrazolium; ROS, reactive oxygen species; RH, relative humidity; $O_2^{\bullet-}$, superoxide.

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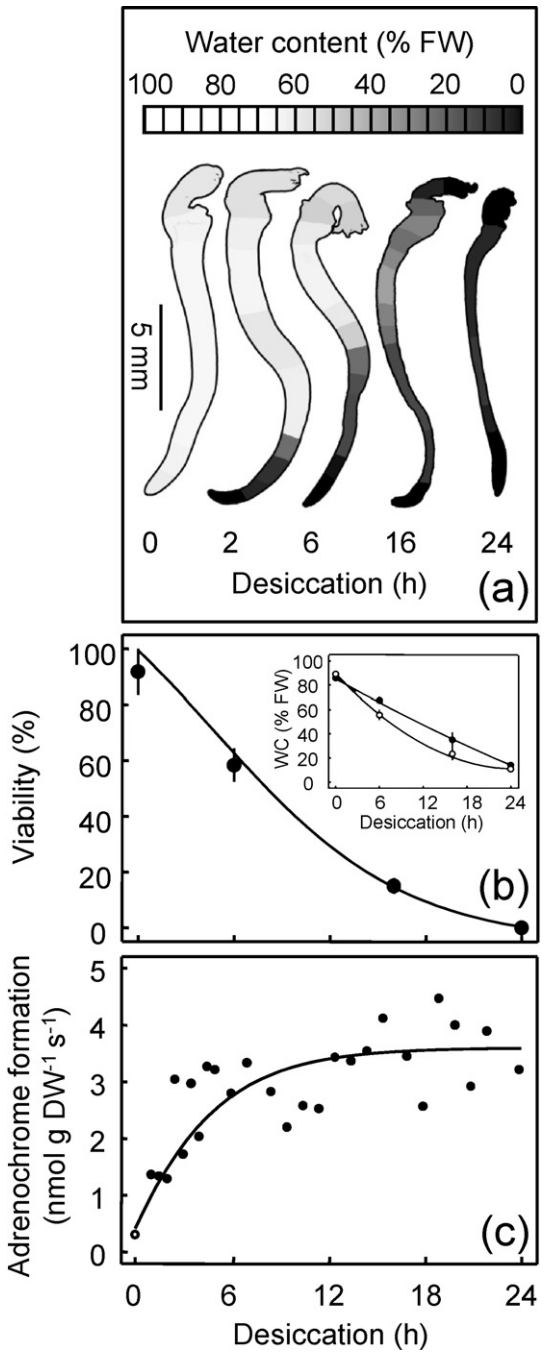


**Fig. 1.** Seedling desiccation, viability and superoxide production. (a) Distribution of water during desiccation. Seedlings were desiccated over silica gel for up to 24 h, then cut into *ca.* 2 mm sections, of which the water content was determined. (b) Seedling viability, expressed as a percentage of seedlings that were able to grow after desiccation followed by rehydration and 21 d of culturing. Data are mean ± SE, $n = 3$ reps of 20 seedlings. The inset shows the WC of hypocotyls (closed symbols) and radicles (open symbols) separately and data are mean ± SE, $n = 10$ seedlings. (c) Extracellular $O_2^{\bullet-}$ production, as measured by adrenochrome formation during 30 min of incubation of seedlings in 1 mM epinephrine, $n = 5$ seedlings per measurement.

Desiccation led to an immediate increase in the rates of extracellular $O_2^{\bullet-}$ production by the seedlings (Fig. 1c). In contrast, desiccating embryonic axes excised from seeds before germination did not induce $O_2^{\bullet-}$ production (data not shown). After 16 h of desiccation, viability dropped to 15 ± 3%, and $O_2^{\bullet-}$ production reached maximal rates and then stayed at this level. Similarly, $O_2^{\bullet-}$ production rates increased during the desiccation of "recalcitrant" (desiccation-sensitive) *Castanea sativa* embryonic axes, although in the recalcitrant system, they declined before major viability loss (Roach et al., 2008, 2010). A direct comparison between the seedling stage of *P. sativum* and embryonic axes of *C. sativa* is limited because the water contents (WC) of fully imbibed *P. sativum* seedlings (Fig. 1b) were *ca.* 30% greater than those of *C. sativa* embryonic axes (Roach et al., 2008). As turgor is required for cell expansion, seedlings are more sensitive to low RH. In *P. sativum*, $O_2^{\bullet-}$ production in desiccated, dying tissues may have originated from events associated to necrosis or programmed cell death (Hengartner, 2000), which has been observed in dehydration-stressed seedlings (Faria et al., 2005).

### *Localization of $O_2^{\bullet-}$ production and putative enzymes involved in its production*

In non-desiccated seedlings, $O_2^{\bullet-}$ production was concentrated at the radicle region above the meristem (Fig. 2a, left image). This is a region in which ROS participate in the signaling implicated in root growth (Krieger-Liszkay et al., 2004), in agreement with the suggested roles of ROS in radicle protrusion during germination, root elongation growth, root hair formation, development and pathogen defense (Kranner et al., 2010b). After 2 h of dehydration (Fig. 2a, centre image) the desiccated root meristem and elongation zone had perished, but the $O_2^{\bullet-}$-producing machinery in the cell walls was apparently still operative (Fig. 2a). The hydrated root region above the meristem was now responsible for 47% of total $O_2^{\bullet-}$ production ($n = 5$ replicates of 5 seedlings split into desiccated and non-desiccated parts). On rehydration, secondary roots were formed in this region of strongly enhanced $O_2^{\bullet-}$ production (Fig. 2a). Therefore, enhanced $O_2^{\bullet-}$ production could be implicated in the signaling required for the establishment of secondary roots. Increased cellular $H_2O_2$ levels have been associated with the emergence of bud primordia in strawberry cultures (Tian et al., 2003), also suggesting a role for ROS in signaling during *de novo* meristem development. We tested two inhibitors of enzymes that could be involved in $O_2^{\bullet-}$ production in the remaining viable section of the radicle. Inhibiting both ECPOX and NAD(P)H oxidases significantly ($P < 0.05$) decreased $O_2^{\bullet-}$ production (Fig. 2b), suggesting that these two enzymes are involved in $O_2^{\bullet-}$ production following desiccation stress. However, further investigations are required to unambiguously identify the enzymes involved in $O_2^{\bullet-}$ production in response to root desiccation.

### *The effect of $H_2O_2$ on post-dehydration shoot and root survival*

After 2 h of dehydration, 56% of the less developed seedlings with short radicles (<3 mm) survived when cultured for 21 d, whereas less than 10% of the more developed seedlings with longer radicles (>16 mm) survived. This finding is in agreement with previous papers reporting that desiccation tolerance can be retained by very young seedlings, but not by more matures ones (Buitink et al., 2003; Faria et al., 2005). However, a 30 min post-desiccation treatment with 10 mM $H_2O_2$ significantly averted viability loss in the more mature seedlings, but not in the less developed seedlings with high initial viability (Fig. 2c). All seedlings were surface-sterilized before culturing to minimize pathogen growth. Therefore, the beneficial roles of $H_2O_2$ in promoting seedling viability were unlikely related to protection from pathogen attack. Similarly, it was reported that $H_2O_2$ can ameliorate desiccation stress in *C. sativa* embryonic axes (Roach et al., 2010). In addition, $H_2O_2$ was required for adventitious root development in mung bean and cucumber and removing endogenous $H_2O_2$ with ascorbate curtailed new root formation (Li et al., 2007; Huang et al., 2011).