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Effects of elevated temperatures on germination and subsequent seedling vigour in recalcitrant *Trichilia emetica* seeds

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ABSTRACT

An increase in atmospheric temperature, particularly in tropical regions, appears to be an inevitable consequence of global climate change. This is likely to affect plant diversity and performance, owing to the relationship between temperature and seed germination, and temperature and plant vigour. The aim of the present study was to investigate the potential effects of elevated temperatures on seed germination and subsequent seedling vigour in a recalcitrant-seeded (desiccation sensitive) tropical African forest species, *Trichilia emetica*. Freshly harvested *T. emetica* seeds were grown at ambient (24 ± 2/14 ± 2 °C) and elevated (29 ± 2/20 ± 2 °C) midday/night temperatures and monitored for germinability, respiratory activity, reactive oxygen species (superoxide and hydrogen peroxide) production, total antioxidant activity and ultrastructural integrity. Seedlings subsequently produced were subjected to various growth measurements to compare vigour between those grown at ambient and elevated temperatures. The results suggest that exposure to elevated temperatures (~5 to 6 °C above ambient) did not disrupt metabolic and ultrastructural integrity in *T. emetica* embryonic axes and consequently did not compromise seed germination and subsequent seedling production. Provided that sufficient water is made available, elevated temperatures may even hasten germinative development in *T. emetica* seeds and improve the competitive ability of seedlings subsequently produced by enhancing seedling growth rates, leaf area and biomass allocation to aerial parts of the plant.

1. Introduction

Global temperatures have increased by 0.6 °C over the past 100 years (Walther et al., 2002) but more marked changes have been noted on a regional scale (Petchey et al., 1999). Global climate models predict a 2–5 °C rise in temperature throughout tropical Africa over the next 50–100 years (Intergovernmental Panel on Climate Change [IPCC], 2007) and this is likely to be accompanied by changing rainfall patterns, changes in seasonality and an increase in the frequency of severe storm events (Seimon et al., 2011). Even though tropical regions in general and Africa in particular are anticipated to experience significant climatic changes (IPCC, 2007), the impacts of these changes on vegetation in these regions have rarely been specifically considered (Scheiter and Higgins, 2009). Predictive models do, however, suggest that areas such as South Africa are likely to face a 5–10% reduction in mean annual precipitation and even though rainfall predictions are less consistent than for temperature (Van Jaarsveld and Chown, 2001), vegetation in these areas is likely to be affected by both increased temperature and rainfall variability (Seimon et al., 2011).

Globally, abiotic stresses associated with climate change have been shown to affect plant cultivation and productivity in a number of crop species, with many of these adopting a series of strategies to reduce the effects of stresses such as elevated temperature and water deficit (Han et al., 2009). More specifically, elevated temperatures have been shown to influence seed germinability, in terms of viability and vigour, and/or seedling establishment in herbaceous (e.g. rice [Han et al., 2009]) and woody (e.g. *Acacia sieberiana* and *Acacia gerrardii* (Mucunguzi and Oryem-Origa, 1996; Chidumayo, 2008)) species. However, our knowledge of the effects of elevated temperatures on seeds and seedlings is almost exclusively based on species that produce orthodox (i.e. desiccation tolerant) seeds (e.g. Baskin and Baskin, 1988 and the references therein). This is alarming since an increasing number of African species are being shown to produce recalcitrant seeds (Berjak and Pammenter, 2008). Recalcitrant seeds, unlike orthodox seeds, are not desiccation tolerant, and are shed at high water content, remaining metabolically active throughout their pre- and post-harvest development (Pammenter and Berjak, 1999).

Very little is known about the effects of measured environmental conditions on germination and seedling establishment in recalcitrant seeds, other than their well-documented desiccation sensitivity (Pammenter and Berjak, 1999; Berjak and Pammenter, 2008). Given their desiccation sensitivity, recalcitrant, unlike orthodox seeds have to germinate shortly after shedding (or else die) and do not remain dormant within natural seed banks (Berjak and Pammenter, 2008). Since the seed's responses to its parental environment are governed by its genetic, physical, physiological and biochemical traits (Daws et al., 2004), the measured and projected increase in temperature (Walther et al.,

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2002) could have serious consequences on recalcitrant seed germination and seedling recruitment within natural habitats. This possibility is further strengthened by the fact that recalcitrant-seeded species are naturally in greatest abundance in sub-tropical and tropical regions (Berjak and Pammenter, 2008), which have already experienced a significant rise in atmospheric temperature (IPCC, 2007).

Unlike other abiotic stresses, like water deficit and salinity, stresses associated with elevated temperature occur within hours (Gür et al., 2010). The unabbreviated post-harvest development of recalcitrant seeds may therefore demand a response to elevated temperatures, be it beneficial or detrimental to seedling establishment. The parental environment influences seed germinability and eventual seedling recruitment (Gutterman, 2000), and if elevated temperatures prohibit recalcitrant-seeded species from establishing seedlings, this could seriously threaten the persistence of wild populations of such species.

The array of wild recalcitrant-seeded species that are likely to be affected by climate change scenarios such as elevated temperature includes woody species of medicinal and economic importance such as *Trichilia emetica* Vahl subsp. *emetica* (Kioko et al., 2006). *T. emetica* grows in forests along the eastern coast of southern Africa and in open riverine-alluvial lowland rainforests of tropical Africa (Beentje, 1994). This woody tree species has a wide range of uses throughout its range, including the use of the bark in herbal medicine, the provision of wood products and the use of oil from the seeds as food and for cosmetic purposes (Grundy and Campbell, 1993). Deforestation together with unsustainable harvesting for traditional medicines threatens the existence of many tropical recalcitrant-seeded species (Grundy and Campbell, 1993). *T. emetica* is presently not threatened but the available populations in South Africa are largely those planted in cities since wild populations are rare (Kioko et al., 2006). Long-term germplasm storage methods are also presently not in place for the species (mainly due to its recalcitrant seeds) and like many other tropical forest species, there are no published data on *T. emetica* seed or seedling responses to climate change. Hence, this study investigated the effects of elevated temperatures on germination and subsequent seedling vigour in *T. emetica* seeds.

In this regard, the exposure of plants and their propagules to abiotic stresses such as elevated temperature can lead to the enhanced production of potentially harmful reactive oxygen species (ROS) (Pukacka and Ratajczak, 2005) and reduced antioxidant capacity (Gür et al., 2010). The oxidative metabolism of a plant/seed under stress can therefore represent a reliable indicator of the severity of the abiotic stress and the organism's ability to respond to the stress (Varghese et al., 2011). Interestingly, ROS have also been implicated in cell wall loosening and radical protrusion during seed germination, suggesting their dual role in seeds (Bailly, 2004; Müller et al., 2009). Additionally, since ROS are agents of intracellular signalling in hydrated tissues, they are likely to play this role in hydrated recalcitrant seeds (Bailly, 2004). Based on the above, in the present study, various measures of oxidative metabolism were used to interpret *T. emetica* germination responses to elevated temperatures. The effects of elevated temperatures on *T. emetica* seed germination were also related to embryonic axis respiratory activity and ultrastructural integrity. Seedlings subsequently produced were subjected to various growth measurements to compare vigour between those growing at ambient and elevated temperatures.

2. Materials and methods

2.1. Seed collection and experimental design

Mature seeds were collected from *T. emetica* trees growing in St. Lucia, KwaZulu-Natal, South Africa (28° 22″ 34′ S 32° 24″ 45′ E). After harvesting, the seeds were immediately conveyed to the laboratory and sown, one per cell and to a depth of ~15 mm, with intact aril in polystyrene seedling trays filled with commercial potting soil (Grovida, South Africa). Soil was watered to field capacity with distilled water prior to this. After sowing, seedling trays were placed in plastic containers with distilled water such that the base of the trays remained submersed for the duration of the experiment (30 days). Maintaining soil moisture at field capacity avoided the imposition of water stress, which can interact with the effects of temperature (Kebreab and Murdoch, 1999).

Four replicates of 200 seeds each were then transferred into a greenhouse in Durban, South Africa for the ambient temperature treatment (midday/night average over 30 days: 24 ± 2/14 ± 2 °C). In order to expose the seeds to elevated temperatures (midday/night average over 30 days: 29 ± 2/20 ± 2 °C), four replicates of 200 seeds were placed in a 6 m by 3 m polyhouse with 0.3 m long by 0.1 m wide air vents on all four sides. This polyhouse was constructed within the greenhouse using 6 mm thick polyethylene greenhouse film (AT Plastics, USA). All eight replicates of 200 seeds each, four at each temperature (viz. ambient and elevated), were treated concurrently for 30 days. As light intensity and humidity in the poly- and greenhouse were comparable when measured at regular intervals during the experiment (using a Kestrel® 4500 Pocket Weather Tracker, USA), these were not manipulated. Air temperature was measured daily within both structures, at 12H00 and 22H00 (Appendix).

Twenty-five cells in each of the eight replicate trays were then labeled and the seeds within them were used for all subsequent germination and seedling growth assessments. Seeds were assessed for signs of germination and seedling emergence 3, 5, 7 and 10 days after planting (DAP), and as >50% germination was attained in both treatments 10 DAP, seeds were thereafter only sampled 24 and 30 DAP to accommodate for post-germination mortalities and delayed germination. The remaining seeds (175 in each replicate tray) were used for biochemical and ultrastructural studies. The sampling days for the biochemical assays (described below) were designed to coincide with the period leading up to, and immediately following, the attainment of ≥50% germination in both treatments (0, 1, 2, 3, 5, 10 and 20 DAP). On each sampling day, sub-samples of seeds (replicate numbers given below) were randomly selected from across all four replicate trays within a treatment and assayed for embryonic axis respiratory activity, extracellular superoxide and hydrogen peroxide production, and total antioxidant capacity. Ultrastructural integrity of root meristematic cells was assessed on day 0, when germination was first observed in both treatments (5 DAP) and upon the attainment of ≥50% germination in both treatments (10 DAP).

2.2. Germination and seedling production

Four replicates of 25 seeds were assessed for germination totality (i.e. percentage of seeds showing radicle protrusion >5 mm) on each of the sampling days and at the end of the experiment for seedling production (i.e. percentage of seeds that produced a root and shoot). *T. emetica* seeds are quite large (>150 mm in length and >50 mm at their widest point) and as they were buried in a pine bark-based potting mix that does not become very compacted after watering, they were easily removed (using a pair of forceps) and reburied for the germination assessments. The germination data was used to calculate the velocity of germination ('germination energy') which as in other studies (e.g. Willan, 1985) was based on the number of days required to reach 50% of the final germination percentage.

2.3. Embryonic axis respiratory activity

On each sampling day, four replicates of one embryonic axis each were stained for viability, and subsequently measured for respiratory

activity using the 2,3,5-triphenyl tetrazolium chloride (TTC) test (Verleysen et al., 2004). The axes were incubated individually in 2 ml of 1% (w/v) TTC, prepared in 0.05 M phosphate buffer (pH 7.3), for 24 h in the dark at room temperature. Tetrazolium chloride reduced to the insoluble red coloured triphenyl formazan, considered to be a positive indication of viability, was thereafter extracted from the tissue in 1 ml of 100% ethanol for 24 h. Respiratory activity was estimated using the absorbance of this extract at 500 nm (Verleysen et al., 2004). The embryos were thereafter dried in an oven (Gallenkam Incubator 1H150, England) at 80 °C for 72 h. Respiratory activity was calculated using the extinction coefficient, 12.2 mM^{-1} cm^{-1}, and expressed as μM formazan g^{-1} dry weight (DW).

2.4. Estimation of superoxide

On each sampling day, four replicates of three embryonic axes each were immersed in 2 ml of 1 mM epinephrine (pH 7) in the dark (at ~25 °C) and shaken at 70 rpm for 15 min (after Misra and Fridovich, 1972). The NADPH-oxidation of epinephrine to adrenochrome was measured as the change in absorbance at 490 nm. The embryos were then dried for 72 h in an oven at 80 °C. The levels of superoxide ($^{\bullet}O_2^-$) produced were calculated using the extinction coefficient, 4.47 mM^{-1} cm^{-1}, and expressed as nmol min^{-1} g^{-1} DW.

2.5. Estimation of hydrogen peroxide

Extracellular hydrogen peroxide (H_2O_2) production was determined according to Gay and Gebicki (2000) using xylenol orange. On each sampling day, four replicates of three embryonic axes each were shaken in 2.0 ml of the working solution (prepared by mixing 0.3 ml of reagent A [25 mM $FeSO_4$, 25 mM $(NH_4)_2SO_4$ and 2.5 M H_2SO_4] and 30 ml of reagent B [125 mM xylenol orange and 100 mM sorbitol], 20 min before use) for 30 min in the dark at 70 rpm. Thereafter, absorbance of the Fe-XO complex produced was measured at 560 nm. The axes were then dried for 72 h in an oven at 80 °C. The amount of H_2O_2 produced was calculated using the extinction coefficient, 267 mM^{-1} cm^{-1}, and expressed as pmol min^{-1} g^{-1} DW.

2.6. Total antioxidant activity

On each sampling day, total antioxidant activity (TAA) was measured for four replicates of 10 embryonic axes each using the 2,2′-azino-bis-3-ethylbenzthiazoline-6-sulphonic acid (ABTS) radical cation decolourisation assay (Re et al., 1999). Axes were transferred to a pre-chilled pestle and mortar, and ground finely in liquid nitrogen with 50 mg of insoluble polyvinyl pyrrolidone. The total antioxidants were extracted in 1 ml of chilled extraction buffer (50 mM potassium phosphate buffer containing 1 mM $CaCl_2$, 1 mM KCl, and 1 mM EDTA; pH 7.0). The homogenate was transferred into a pre-chilled Eppendorf tube and centrifuged at 14,000 rpm at 4 °C for 30 min. The resulting supernatant was extracted and kept on ice. The ABTS solution (containing 7 mM ABTS and 2.45 mM potassium persulphate in 1 ml of distilled water) was prepared 12–16 h prior to the assay and kept in the dark. This ABTS solution was diluted with phosphate buffered saline (PBS; pH 7.4) until an initial absorbance of ~0.72 at 734 nm was achieved. The decolourisation of the working solution was measured 2 min after the addition of 10 μl of the antioxidant extract. A standard curve, using 0.05–1.0 mM Trolox™ (Sigma-Aldrich, Germany) dissolved in PBS, was constructed and the change in absorbance due to the tissue antioxidant extract was expressed as Trolox equivalents on a fresh mass basis using this curve.

2.7. Ultrastructural studies

On 0, 5 and 10 DAP the radicle tip, containing the root meristem, was excised from five embryonic axes and fixed for transmission electron microscopy (TEM). Primary fixation was carried out in 2.5% glutaraldehyde for 24 h at 4 °C. Samples were post-fixed in 0.5% aqueous osmium tetroxide and subsequently dehydrated in a graded acetone series before infiltration with resin. Fixed specimens were then sectioned using an ultra microtome (Ultracut-E, Leica, Austria) and ultra-thin copper/gold sections (0.1 μm) of root meristem cells were stained using uranyl acetate followed by lead citrate. These sections were viewed using a Jeol JEM 1010 transmission electron microscope (JEOL, Japan) at 100 kV. The ultrastructure of root meristem cells from seeds germinating at both temperatures was assessed and captured digitally for subsequent analysis.

2.8. Seedling growth and biomass

Thirty-one DAP, 10 seedlings were randomly selected from each of the four replicate trays within a treatment ($n = 40$), measured for root and shoot length, and thereafter individually separated into roots, shoots and leaves. The cumulative leaf area of each seedling was then measured using a leaf area meter (C1-202 Area Meter, CID Inc., USA). Thereafter, the leaves, roots and shoots were oven-dried at 80 °C for 72 h prior to dry weight estimation.

2.9. Statistical analysis

The data was analysed using PASW 18 statistics version 18.0.3 (SPSS Inc., Chicago, Illinois, USA). In order to determine whether there were any differences in germination and seedling production between treatments, percentiles were arcsine transformed before subjecting the data to analysis of variance (ANOVA). ANOVA was also used to test for inter-treatment differences in germination velocity, axis superoxide and hydrogen peroxide production, respiratory rate, total antioxidant capacity, and seedling leaf area, biomass production and allocation. Where possible, means were separated using a Tukey post-hoc test and differences were considered significant at the 0.05 level.

3. Results and discussion

Temperature, along with other factors that cause climatic variation, can influence seed germination and seedling recruitment (Walck et al., 2011). This, together with anthropogenic changes such as land clearing, is predicted to impact on vegetation types across southern Africa, including indigenous forest (Eeley et al., 1999). The potential effects of climate change scenarios such as elevated temperature on recalcitrant-seeded species are therefore an important ecological consideration, as seeds of these species are desiccation-sensitive, do not display dormancy and hence, do not persist in natural seed banks (Daws et al., 2004). This motivated the present study on the effects of elevated temperatures on germination and subsequent seedling vigour in a recalcitrant-seeded African forest species, *T. emetica*.

3.1. Seed responses to elevated temperatures

3.1.1. Germination totality and velocity

Temperature regulates germination by determining the totality and rate of germination, by removing primary and/or secondary dormancy, and by inducing secondary dormancy (Bewley and Black, 1994). In the present study, exposure of *T. emetica* seeds to a ~5 to 6 °C increase in day and night atmospheric temperatures did not compromise germination significantly relative to ambient temperatures ($p > 0.05$; final germination >80% in both treatments; Fig. 1). When embryonic axes were excised from non-germinated seeds in both treatments at the

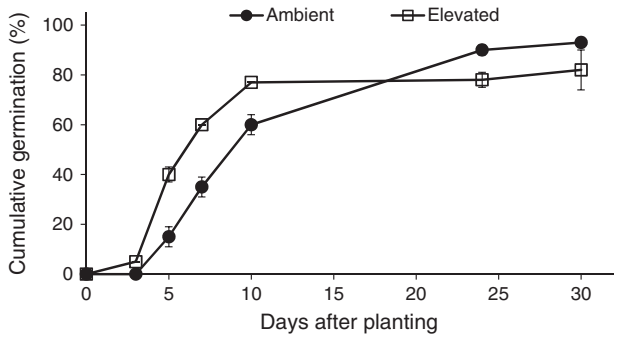

Fig. 1. Effects of 30 d exposure to ambient (●) and elevated (□) temperatures on *T. emetica* seed germination capacity. Values represent the mean ± SD of 4 replicates of 25 seeds each and data are shown as cumulative germination: percentage germination on any particular day after planting, added to the value from previous observation days. $p > 0.05$ when values for 30 DAP were compared between treatments (ANOVA).

end of the experiment, these showed no signs of radicle elongation and did not stain positively with TTC. This suggests that their failure to germinate was more likely a consequence of seed mortality than delayed or aborted germination. In general, increasing temperature has been found to increase total germination percentage and germination rate but exposure to any temperature beyond the optimum range for germination can negatively affect seed germination (Finch-Savage and Leubner-Metzger, 2006). There were no published reports on the effects of elevated temperatures on germination in recalcitrant seeds at the time of this study but research on a limited of orthodox-seeded southern African tree species suggests that whilst a moderate increase in temperature promotes seed germination in some species, a further increase almost always causes seed mortality (Mucunguzi and Oryem-Origa, 1996; Chidumayo, 2008).

Whilst germination (i.e. the percentage of the seeds that have germinated during the test period) is essential when assessing the effects of a treatment on seed viability, it is also important to assess the velocity of this germination since rapid germination is an advantage for seedling establishment under field conditions (Parsons, 2012). Velocity of germination, also termed 'germination energy', is an expression of seed vigour (Willan, 1985) and in this study was significantly higher in *T. emetica* seeds exposed to elevated as opposed to ambient temperatures: 5 ± 1 d required to reach 50% of the final germination percentage at elevated temperatures, compared with 9 ± 1 d at ambient temperatures ($p < 0.05$; ANOVA). Additionally, the data presented in Fig. 1 shows that cumulative germination was initially (3–10 DAP) higher at elevated, as opposed to ambient temperatures. However, whilst cumulative germination continued to increase at ambient temperatures beyond day 10, it almost levelled-off at elevated temperatures. Seeds of different species respond differently to temperature changes (Walck et al., 2011) and seedling emergence is known to be synchronized with changes in the environment (Baskin and Baskin, 1988). The speed of germination has important ecological significance and any environmental factor that alters a species characteristic speed of germination, has the potential to influence the persistence of that species in natural habitats (Parsons, 2012). The results obtained here suggest that elevated temperatures may enhance rate of germination in *T. emetica* seeds, which may place them at an ecological advantage or disadvantage, based on whether or not environmental conditions support seedling establishment after radicle protrusion.

3.1.2. Respiratory activity

In order to interpret the germination totality and velocity data discussed above, we measured respiratory activity in embryonic axes excised from *T. emetica* seeds exposed to ambient and elevated temperatures. Respiration, or the uptake of oxygen, is one of the most crucial metabolic factors necessary for successful germination of seeds (Wang et al., 2012). When respiratory activity is impaired, a reduced amount of oxygen is taken up by seed tissues and this is known to cause seed deterioration in species like soybean (Leopold, 1980) and cotton (Woodstock et al., 1985). The results obtained here (Fig. 2) showed no inter-treatment differences in respiratory activity, except on day 2, when respiratory rates of seeds at ambient temperatures were significantly ($p < 0.05$) higher than those at elevated temperatures. This difference on day 2 cannot be explained at present but respiratory rates in both treatments generally appeared to increase until day 5, after which they declined. Recalcitrant seeds are shed fully hydrated and metabolically active and begin germinative development immediately if conditions permit (Pammenter and Berjak, 1999). As water content increases, respiration rates increase to sustain embryo development (Mylona et al., 2007) and this may explain the progressive increase in respiratory rate, up until day 5, in seeds from both treatments. Additionally, respiratory activity is one of the major sources of ROS in seeds, making the mitochondria the most sensitive organelle to abiotic stress (Miller et al., 2007). The lack of inter-treatment differences in *T. emetica* axis respiratory rates suggests that any inter-treatment differences in ROS production (to be discussed next) may not have been a product of differences in respiratory rates but rather part of a biochemical signal for germination. This suggestion is based on a recent study on recalcitrant *Trichilia dregeana* Sond. seeds which showed that ROS may form part of the biochemical trigger for germination when the seeds are exposed to slight dehydration (Varghese et al., 2011).

3.1.3. Oxidative metabolism

Leading on from above, extracellular $^{\bullet}O_2^-$ production remained relatively comparable between seeds exposed to ambient and elevated temperatures up until day 5 after which differences in $^{\bullet}O_2^-$ production were noted in seeds from both treatments (Fig. 3). However, the peak in $^{\bullet}O_2^-$ production occurred much earlier in seeds exposed to elevated temperatures and this may explain the difference in the rate of germination between the two treatments (Fig. 1). The peak in $^{\bullet}O_2^-$ production which coincided with maximum germination at both temperatures could be representative of the burst of ROS typically accompanying germination in seeds (El-Maarouf-Bouteau and Bailly, 2008). Superoxide is considered to be critical for vigorous germination (Schopfer et al., 2001; Liszkay et al., 2004). For example, studies have shown that the inhibition of elongation in maize seedling roots was accompanied by a decreased rate of $^{\bullet}O_2^-$ production (Liszkay et al., 2004).

Hydrogen peroxide production was also measured in this study (Fig. 4), and except for day 2 remained comparable between treatments, peaking on day 20, when already formed radicles in the bulk of seeds

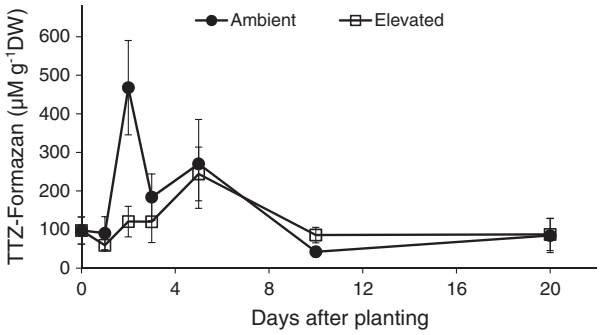

Fig. 2. Effect of ambient (●) and elevated (□) temperatures on embryonic axis respiratory activity in *T. emetica* seeds. Values represent the mean ± SD, $n = 4$ ($p < 0.05$ for day 2; $p > 0.05$ for other sampling days; ANOVA).

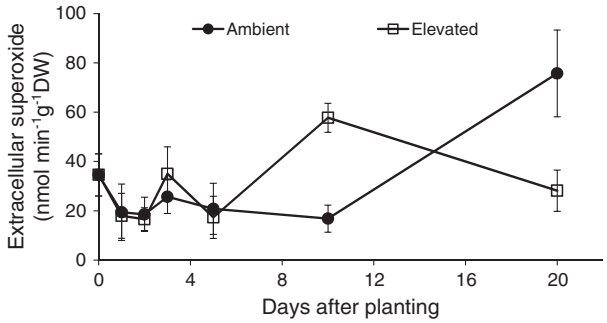

Fig. 3. Effect of ambient (●) and elevated (□) temperatures on *T. emetica* embryonic axis extracellular superoxide production. Values represent the mean ± SD, $n = 4$ ($p < 0.05$ for days 10 and 20; $p > 0.05$ for other sampling days; ANOVA).

within both treatments were elongating (i.e. growing). Relatively higher levels of H_2O_2 observed in seeds at elevated temperatures 2 DAP may be reflective of the enhanced ROS production associated with axis elongation following imbibition (Fig. 4). Gidrol et al. (1994) have also shown that high H_2O_2 levels occur during early stages of imbibition in soybean seeds. The possible involvement of H_2O_2 in the elongation phase following imbibition in *T. emetica* seeds is further supported by the fact that H_2O_2 levels in seeds at ambient temperature rose noticeably on day 3, from relatively low levels on day 2. Hydrogen peroxide has been shown to be a key regulator in a broad range of physiological processes, including germination, which it has been shown to promote in a dose dependent manner (Schopfer et al., 2001; Barba-Espín et al., 2011).

The ROS data obtained here supports the view that seed germination is accompanied by the generation of ROS in embryonic axes (Schopfer et al., 2001) and suggests that the timing of this generation may be hastened in *T. emetica* seeds by exposing them to elevated temperatures. Indications that germination associated ROS bursts occurred earlier in seeds exposed to elevated temperatures may explain the enhanced germination rate exhibited by seeds at elevated, compared with ambient temperatures (Fig. 1). This suggestion is supported by the fact that whilst elevated temperatures that impose a stress in orthodox seeds usually induce the production of antioxidants (Baskin and Baskin, 1988), relatively low total antioxidant activities (TAA) were noted for *T. emetica* seeds at elevated temperatures during the initial phase of germination (Fig. 5). These relatively low initial TAA levels in seeds exposed to elevated temperatures suggest a probable deliberate mechanism to allow (i.e. not quench) ROS necessary for cell wall loosening and other growth processes during the early phases of germination. However, since TAA levels did eventually rise on days 5 and 10 to levels comparable with seeds at ambient temperatures (Fig. 5), there is also the possibility that elevated temperatures may have eventually compromised TAA in *T. emetica* seeds. Abiotic stresses (e.g. dehydration and low temperatures) have been shown to compromise antioxidants in recalcitrant seeds and if sustained, can result in viability loss (Varghese et al., 2011) since antioxidants scavenge potentially harmful reactive nitrogen and oxygen species. Conclusive decisions on the applicability of either one of the two hypotheses presented above are not possible at present. This will require more detailed investigations, such as comparison of the responses of the enzymic and non-enzymic components of the antioxidant system to elevated temperatures, rather than an estimation of total antioxidant activity (as carried out here).

3.1.4. Cellular ultrastructure

At this point it is instructive for us to consider the cellular ultrastructure associated with the metabolism that accompanied germination in *T. emetica* seeds exposed to ambient and elevated temperatures. Embryonic axes excised from freshly harvested seeds (day 0) showed well-defined cell walls, many small vacuoles (Fig. 6A and B), and non-spherical nuclei (Fig. 6B). Plastids, though present (Fig. 6A), did not appear to be abundantly filled with starch grains, which is a common feature of actively metabolising recalcitrant seeds (Pammenter and Berjak, 1999). Profiles of endoplasmic reticulae and Golgi bodies were also rare, however, cytomatrical polysome development, indicative of protein synthesis, was apparent (Fig. 6B). As discussed earlier, respiratory activity was measurable in these axes (Fig. 2) and this was corroborated by the presence of 3–4 mitochondria per cell (Fig. 6B). The cells displayed numerous small vacuoles, with contents being noted in some of these (Fig. 6A and B). Recalcitrant seeds continue to be actively metabolic throughout development, this being the major factor in their continuing desiccation sensitivity (reviewed by Pammenter and Berjak, 1999; Berjak and Pammenter, 2008). However, the ultrastructure of the meristematic cells within embryonic axes excised from freshly harvested seeds was not representative of highly metabolic meristematic cells usually seen in embryonic axes that are close to radicle protrusion. Such cells would usually have an abundance of well-developed mitochondria to facilitate cellular respiration, deposition of material within the plastids and abundant Golgi bodies (Pammenter and Berjak, 1999).

Recalcitrant seeds do, however, display a steadily changing metabolic status as they approach and enter germination (Pammenter and Berjak, 1999) and this was evidenced in this study by the ultrastructure of *T. emetica* seeds sampled 5 DAP. On this day, meristematic cells from seeds sown at ambient and elevated temperatures showed ultrastructural signs of active metabolism (Fig. 7A–D), typically associated with

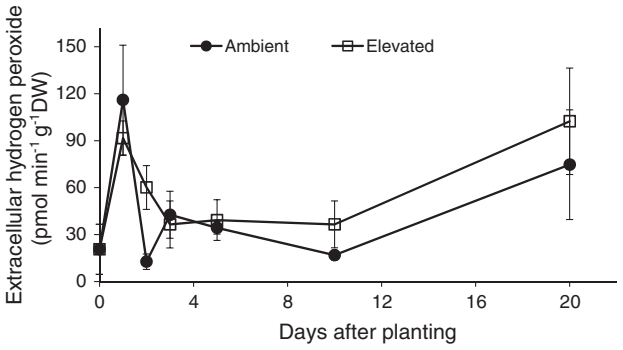

Fig. 4. Effect of ambient (●) and elevated (□) temperatures on *T. emetica* embryonic axis extracellular hydrogen peroxide production. Values are the mean ± SD, $n = 4$ ($p < 0.05$ for day 2; $p > 0.05$ for other sampling days; ANOVA).

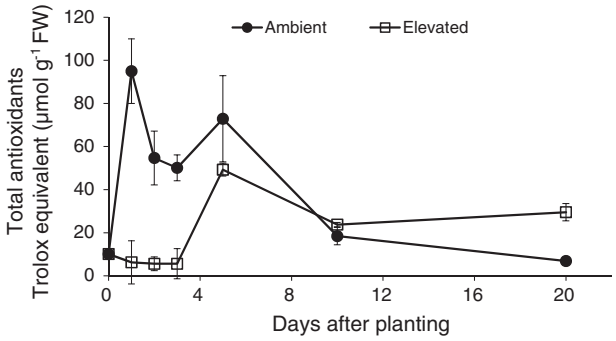

Fig. 5. Effect of ambient (●) and elevated (□) temperatures on *T. emetica* embryonic axis total antioxidant activity. Values are the mean ± SD, $n = 4$ ($p < 0.05$ for days 1, 2, 3 and 20; $p > 0.05$ for other sampling days; ANOVA).

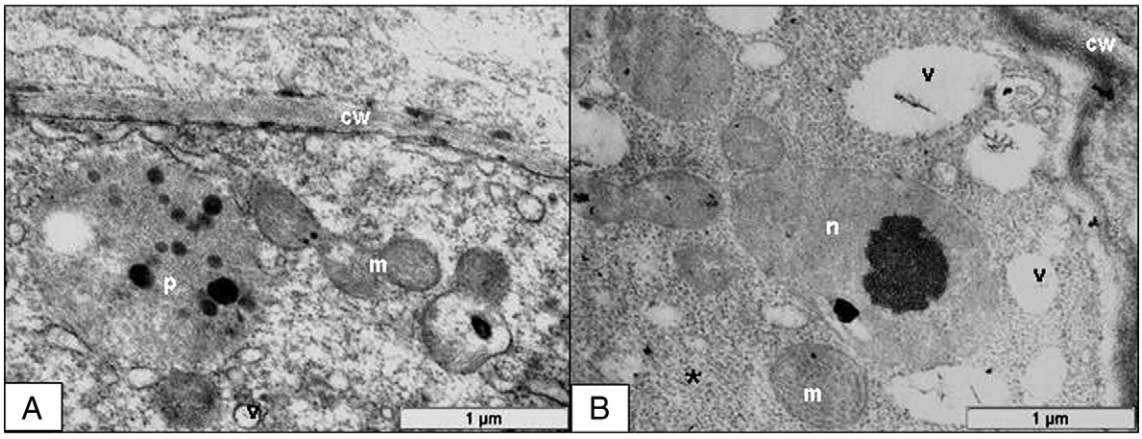

Fig. 6. The ultrastructure of embryonic axes from freshly harvested (control) seeds. Cell walls (cw) were clearly defined and enclosed cells with polysomes (*), cristate mitochondria (m), plastids (p) and small vacuoles (v) with some flocculent contents.

the onset of germination in recalcitrant seeds (Pammenter and Berjak, 1999). Meristematic cells from seeds developing at both temperatures showed well defined cell walls (Fig. 7A and C), and signs of ongoing metabolic activity in terms of the dense cristate mitochondria and abundant polysomes (Fig. 7B and D). Cells of seeds from both temperatures displayed distinct profiles of endoplasmic reticulum (ER) and this suggested functionality of the endomembrane system. Additionally, the fact that the polysomes were markedly more apparent within the cytomatrices of cells from both treatments suggested active protein synthesis (Fig. 7B and D). The appearance of vacuoles containing flocculent material, indicative of cell component turn-over, and considerable deposition of starch within plastids was a new feature of cells from both treatments (Fig. 7B and D). However, closer examination of the ultrastructure revealed slight inter-treatment differences, suggesting that axes from seeds germinating at elevated temperatures were considerably more metabolically active and probably at

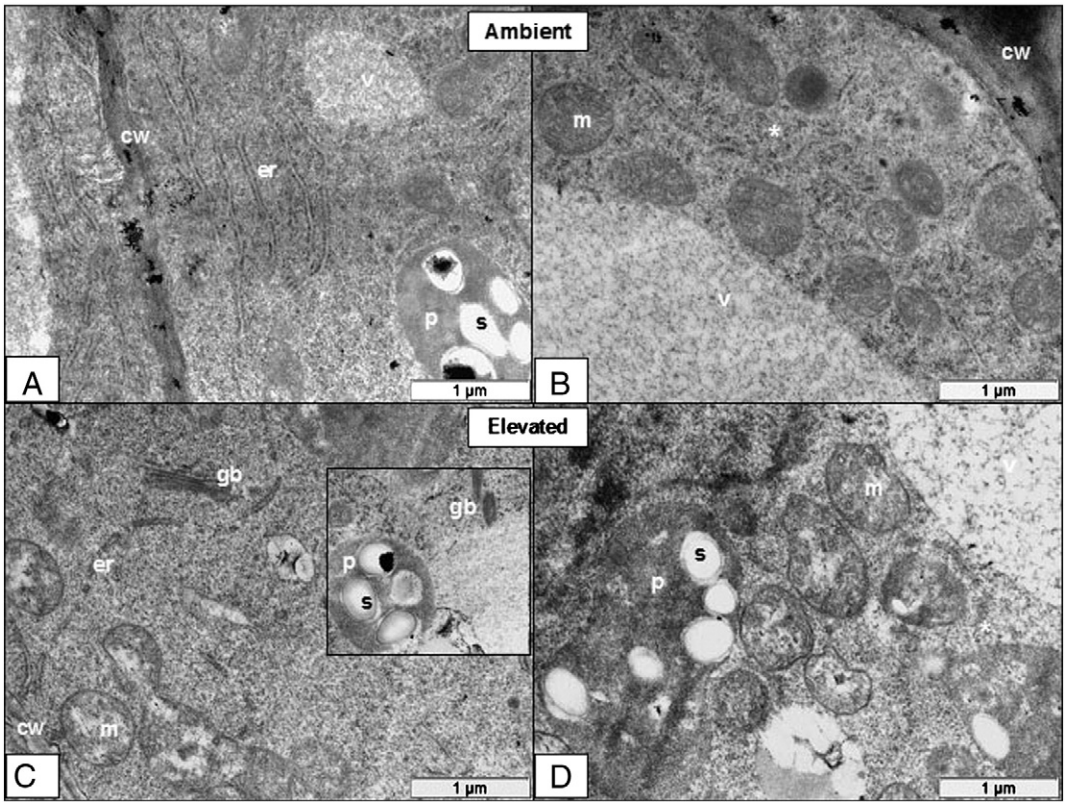

Fig. 7. The ultrastructure of embryonic axes of seeds sown at ambient (A & B) and elevated (C & D) temperatures on 5 d after sowing. Cell walls (cw) were clearly defined and enclosed cells with cristate mitochondria (m), small vacuoles (v) with flocculent contents, starch (s) filled plastids (p), endoplasmic reticulae (er) and polysome-rich (*) cytomatrix. Golgi bodies (gb) were frequent in cells from seeds incubated at elevated temperatures (C) but rare in seeds incubated at ambient temperatures.

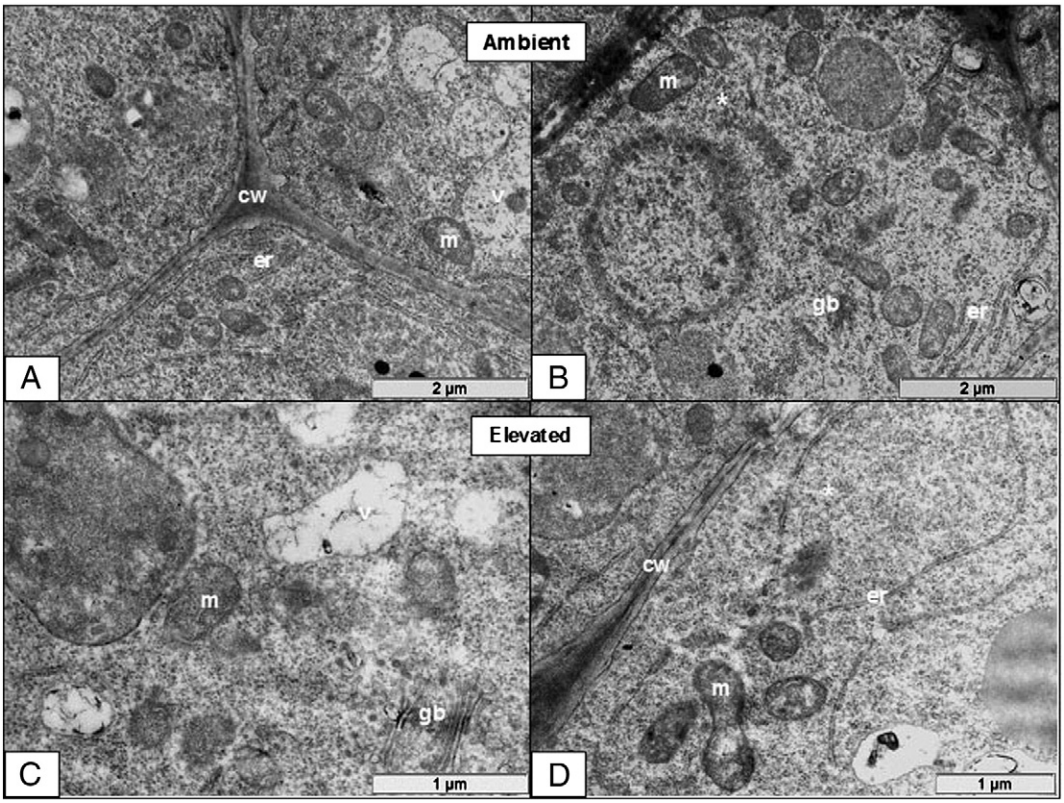

Fig. 8. The ultrastructure of seeds exposed to ambient (A & B) and elevated (C & D) temperatures for 10 d after sowing. Cell walls (cw) were clearly defined and enclosed cells with cristate mitochondria (m), small vacuoles (v) with flocculent contents, and polysome-rich (*) cytomatrix. Golgi bodies (gb) and endoplasmic reticulae (er) were present in cells from seeds incubated at both temperatures.

a more advanced developmental stage, in terms of the events leading up to germination. This was implicit in the relatively greater frequency of starch filled plastids and Golgi bodies in cells from seeds sown at elevated temperatures (Fig. 7C): 1–2 starch filled plastids and 1–3 Golgi bodies in cells from seeds at ambient temperatures versus 2–4 starch filled plastids and 3–5 Golgi bodies in cells from seeds at elevated temperatures. These observations suggest that exposure to elevated temperatures does not disrupt germinative metabolism or compromise ultrastructural integrity in meristematic cells within *T. emetica* embryonic axes and support the germination and biochemical data presented earlier. In fact, the ultrastructural comparisons between cells from seeds sown at ambient and elevated temperatures suggest that the latter may actually have heightened germinative metabolism in *T. emetica* seeds, which may also explain the higher germination velocity displayed by these seeds at elevated temperatures (Fig. 1).

By day 10, cells from seeds at both temperatures possessed an ultrastructure typical of an enhanced state of metabolism (Fig. 8A–D): many dense cristate mitochondria (6–11 per cell) and polysomes (Fig. 8A–D), and distinct ER profiles (Fig. 8B and D) and Golgi bodies (Fig. 8B and C), suggesting functionality of the endomembrane system. Cell walls were well defined and vacuoles were small and not abundant (Fig. 8A and C). Starch containing plastids which were a feature of cells from seeds of both treatments on day 5 (Fig. 7A and D) were no longer evident. The marked decrease in starch deposits could well have yielded sugars necessary for the growth processes accompanying germination. Unlike day 5, there was little evidence that seeds at elevated temperatures were more metabolically active; however, there were no signs of vacuolar fusion, irregular cell walls and cytoplasmic clearing, which have all been identified as signs of incipient deterioration of cells in recalcitrant embryonic axes (Sershen et al., 2012). The absence of these abnormalities in seeds exposed to elevated temperatures corroborates the high viability retention and germination in seeds exposed to elevated temperatures (Fig. 1).

3.2. Seedling responses to elevated temperatures

Having discussed the responses of *T. emetica* seeds to elevated temperatures, it is important to note that whilst germination of seeds within

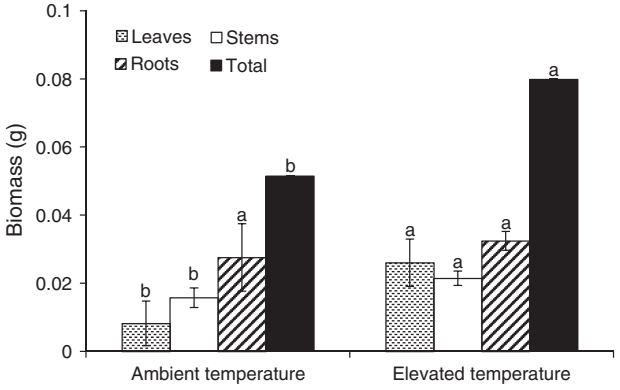

Fig. 9. Biomass production in *T. emetica* seedlings grown under ambient and elevated temperature conditions. Values represent the mean ± SD, $n = 40$. Bars labeled with different letters are significantly different when compared across treatments, within categories (e.g. leaves) ($p < 0.05$; ANOVA).

Table 1
Leaf area and indicators of plant growth performance for *T. emetica* seedlings grown under ambient and elevated temperature conditions for 30 d.

Treatment	Cumulative leaf area (cm^2)	Root:shoot ratio	Leaf area ratio ($cm^2\ g^{-1}$)
Ambient temperature	2.90 ± 1.3	1.85 ± 2.2	62.06 ± 26.4
Elevated temperature	10.84 ± 3.8	1.51 ± 0.3	132.51 ± 25.8

Values are the mean ± SD, $n = 40$ ($p < 0.05$ for cumulative leaf area and leaf area ratio and $p = 0.92$ for root:shoot ratio; ANOVA).

natural seed banks is important, it is the ability of germinated seeds to establish as healthy, vigorous seedlings that will ultimately determine the persistence of natural populations (Grice and Westoby, 1987). As discussed earlier elevated temperatures did not compromise germination in *T. emetica* seeds significantly ($p > 0.05$; Fig. 1) and 30 DAP 82 ± 8% of the seeds at elevated temperatures produced seedlings (root and shoot), which was slightly, but not significantly ($p = 0.07$; ANOVA), lower than the 93 ± 2% achieved for those at ambient temperatures. There is a paucity of information on the seed ecology of both orthodox- and recalcitrant-seeded African species and little information on their responses to rising temperature (Turpie et al., 2002). A few studies on African woody plants do, however, suggest that whilst the seedlings of some species like *Combretum apiculatum* may be able to adapt to high-temperature habitats (Choinski and Tuohy, 1991), extended exposure to high temperatures will induce seedling mortality in others (e.g. some *Acacia* spp. (Chidumayo, 2008)).

In the present study, we compared the vigour of *T. emetica* seedlings produced under ambient and elevated temperature conditions and growth analyses revealed that seedlings produced at elevated temperatures possessed significantly higher stem, leaf and total biomass ($p < 0.05$; Fig. 9). This suggests that elevated temperatures may have improved *T. emetica* seedling growth rates. Growth rates are intimately linked to photosynthetic rates, which in turn depend on leaf area (Ward and Strain, 1999). Studies have shown that elevated temperatures can enhance photosynthetic rates and biomass allocation to leaves (Ward and Strain, 1999). The measurement of photosynthetic rates was beyond the scope of this study. However, significantly ($p < 0.05$) greater leaf area (Table 1) and biomass allocation to leaves (Fig. 10) in *T. emetica* seedlings produced at elevated temperatures suggest that they may have exhibited higher photosynthetic rates than those produced at ambient temperatures. This possibility is reinforced by the fact that leaf area ratio (an indication of the amount of photosynthetic machinery) was significantly greater for seedlings at elevated temperatures ($p < 0.05$; Table 1).

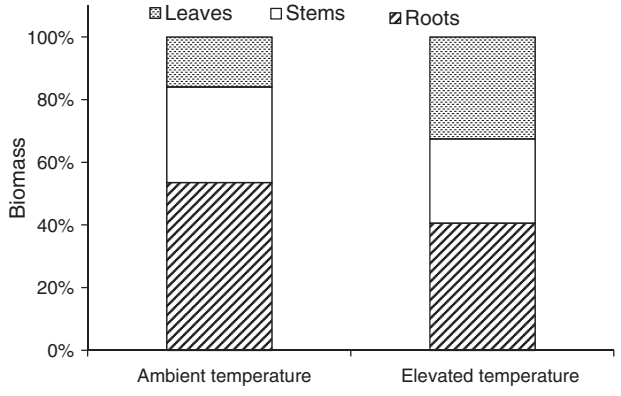

Fig. 10. Biomass allocation in *T. emetica* seedlings produced under ambient and elevated temperature conditions. Values represent the mean, $n = 40$ ($p < 0.05$ for stems and leaves; $p > 0.05$ for roots; ANOVA).

The increased biomass allocation to leaves in seedlings produced at elevated temperatures seems to have occurred at the expense of roots; however, root:shoot ratios (i.e. a reflection of the differential investment of photosynthates between the aboveground and belowground organs) did not differ significantly between temperature treatments ($p = 0.92$; Table 1). These results suggest that elevated temperatures did not compromise the overall health of *T. emetica* seedlings relative to those produced at ambient temperatures and agree with other studies which have found no significant trend observed in the relationship between root:shoot ratios and temperature for forest and woodland taxa (Mokany et al., 2006 and the references therein).

4. Concluding remarks

Contrary to reports for some orthodox-seeded African tree species (e.g. selected *Acacia* spp. (Mucunguzi and Oryem-Origa, 1996; Chidumayo, 2008)) elevated temperatures did not compromise *T. emetica* seed germination (Fig. 1) and seedling production, but enhanced germination velocity (Fig. 1), relative to ambient temperatures. Comparisons of respiratory rates, oxidative metabolism and ultrastructural integrity between seeds exposed to ambient and elevated temperatures also suggest that elevated temperatures may hasten germinative development in the recalcitrant seeds of this African tree species. Provided that sufficient water is available, this increased germination velocity at elevated temperatures may represent an ecological advantage, since the large seeds of many recalcitrant-seeded species like *T. emetica* are usually more susceptible to herbivory and desiccation than small seeds (Howe and Richter, 1982).

Furthermore, the results of the seed and seedling studies discussed above suggest that elevated temperatures may improve the ability of *T. emetica* to compete with neighbouring species during the seedling establishment phase, by enhancing seedling growth rates, leaf area (Table 1) and biomass allocation to aerial parts of the plant (Fig. 10). This is encouraging since competitive interactions occur at high frequencies in natural communities, influencing species distribution patterns, relative abundances, diversity and thus community structure (Goldberg and Barton, 1992). More specifically, interspecific competition for light is often the single most important proximal cause of plant dominance (Baskin and Baskin, 1988). The value of the observations made for *T. emetica* seedlings here is appreciated when one considers that the slow persistent growth of this species in the wild makes it highly susceptible to competition for light from more rapidly growing herbaceous species (Beentje, 1994).

Tropical regions and Africa in particular are anticipated to experience the most significant climatic changes (IPCC, 2007). Given the increasing number of African species that are being shown to produce recalcitrant seeds (Berjak and Pammenter, 2008) and the climatic and anthropogenic threats faced by many of these species (Grundy and Campbell, 1993; Daws et al., 2006), it is becoming increasingly important to investigate the impacts of climate change scenarios on tropical African species such as *T. emetica*. We therefore recommend that this investigation be extended to other indigenous species since knowledge of their seed germination and seedling establishment under various climate change scenarios will be essential for their successful re-introduction into biodiversity-eroded habitats in the future.

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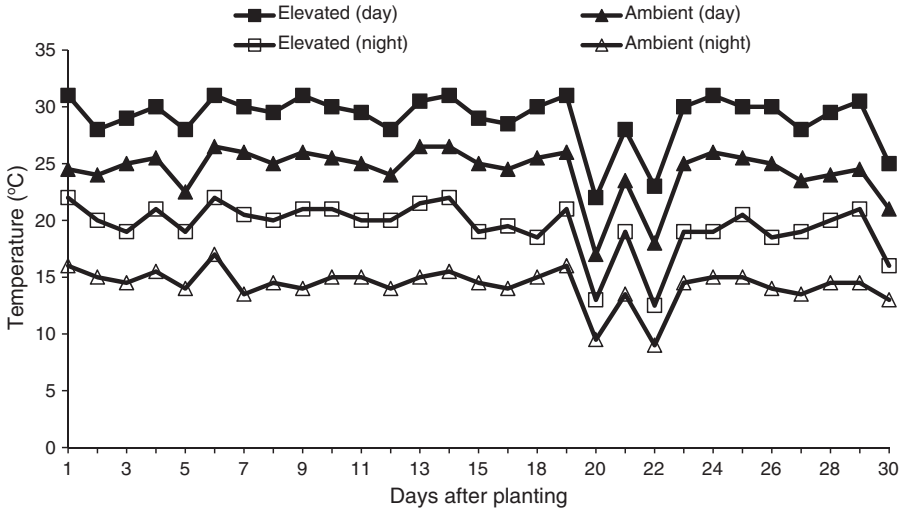
Appendix

Daily midday (12H00) and night (22H00) air temperatures in greenhouse (ambient) and polyhouse (elevated) measured over the 30 d experimental period.



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