Annals of Botany **96**: 159–163, 2005
doi:10.1093/aob/mci151, available online at www.aob.oupjournals.org

SHORT COMMUNICATION

Successful Seed Germination of the Nickel Hyperaccumulator *Stackhousia tryonii*

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Received: 22 November 2004 Returned for revision: 19 January 2005 Accepted: 28 February 2005 Published electronically: 19 April 2005

• *Background and Aims Stackhousia tryonii*, a rare nickel hyperaccumulating herb, is endemic to ultramafic (serpentine) soils of central Queensland, Australia. The effects of eight dormancy-relieving treatments on germination of stored seeds of *Stackhousia tryonii* were investigated under controlled light and temperature conditions.
• *Methods* The treatments were: untreated (control i), leached and dehydrated (primed control ii), treating with gibberellic acid (150 and 300 μM), smoke extract (5 and 10 %, v/v) and potassium cyanide (40 and 80 mM).
• *Key Results* Freshly harvested seeds did not germinate. Germination percentage increased with time of storage for up to 18 months (38·3 %). Gibberellin, smoke extract and cyanide treatments did not significantly improve germination. Light did not affect seed germination and there was no interaction between dormancy-relieving treatments and light. A significant inhibition of germination occurred in seeds treated with 5 % (but not 10 %) aqueous smoke extract. Saturated fatty acids, predominantly tridecanoic ($C_{13:0}$), constituted about 90 % of the total fatty acids in the oil of freshly harvested seeds. In contrast, there was increased accumulation (>75 %) of mono-unsaturated (oleic, $C_{18:1}$) and poly-unsaturated (linoleic, $C_{18:2}$; linolenic, $C_{18:3}$) fatty acids in the oil of stored seeds.
• *Conclusions* Seeds of *S. tryonii* require an after-ripening period for germination.

Key words: After-ripening period, central Queensland, fatty acid, metal hyperaccumulation, nickel (Ni), seed dormancy, seed germination, serpentine soil, *Stackhousia tryonii*, ultramafic soil.

INTRODUCTION

Some higher plants have developed heavy metal tolerance strategies that enable them to survive and reproduce in highly metal-contaminated soils. A very small number of such species (<0·2 % of angiosperms) are absolute metallophytes and are metal hyperaccumulators growing only on metal-enriched substrates (Baker *et al*., 2000).

All metallophytes can potentially be used in the restoration of areas following mining, remediation of metal-contaminated soils and waters (phytoremediation; Baker *et al*., 1994), or phytoextraction of metals for economic returns (phytomining; Nicks and Chambers, 1995). Metallophytes colonizing such soils have limited geographic distribution and are considered as rare and vulnerable to elimination because of their limited occurrence. Therefore, there is an urgent need to conserve such biological resources (Whiting *et al*., 2003).

Stackhousia tryonii (Family Stackhousiaceae, Order Celastrales of the Sub-Class Dicotyledons) is a rare (Briggs and Leigh, 1996), perennial, nickel hyperaccumulator (Batianoff *et al*., 1990; Bhatia, 2003), which is an herbaceous indicator of nickeliferous (ultramafic) soils in central Queensland, Australia. *Stackhousia tryonii* is suitable for rehabilitation of nickel mine tailings and it also possesses a high ornamental value. However, poor and erratic germination (Bhatia, 2003) has limited its exploitation. *Stackhousia* spp. are usually propagated from stem cuttings (Ralph, 1997).

Stackhousia tryonii usually flowers after seasonal rains, and produces a large number of fruits. It is an obligate seeder. A mature plant produces 50–100 branches and sets a few hundred fruits that mature in 4–6 weeks. These are subsequently shed on the ground and appear to remain dormant in the soil seed bank until cued to germinate, mainly following fire.

The fruit is a schizocarp, breaking at maturity into three single-seeded units, each about 2 mm long, consisting of a seed within a carpel. There are no previous reports on propagation of *S. tryonii* via seeds (CAB Abstracts, 1973–2004, CAB International, Wallingford, UK; Ovid Technologies, Sydney, Australia). Generally, seed germination in the Stackhousiaceae is very poor. For example, in *Stackhousia huegelii* and *Tripterococcus brunonis*, Dixon *et al*. (1995) recorded no germination in control and smoke-treated seed lots. However, germination in *S. pubescens* was significantly higher (mean germination = 3·6 %) in smoke-treated seed lots compared with untreated controls. In contrast, Roche *et al*. (1997*a*) recorded up to 48 % germination in seeds of *S. pubescens* following smoke treatment.

Seeds of some species do not germinate when placed under conditions normally regarded as favourable to germination and are said to be dormant (Bewley and Black, 1982). Dormancy is one of the most important

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adaptive properties of wild species, and is a device for optimizing the distribution of germination in time and place (Nikolaeva, 1977; Hilhorst, 1993). Mechanical barriers created by the pericarp and inhibitors may be responsible for the imposition and maintenance of dormancy (Slattery *et al*., 1982). Alternatively, dormancy may be induced by inhibitors of various chemical classes, including phenolics, tannins, waxes and some short-chain saturated acids (Berrie *et al*. 1979; Bewley and Black, 1982). These short-chain saturated carboxylated acids (particularly C1 to C12) inhibit various processes in plants, including germination (Rogoyski and Powell, 1981; Bewley and Black, 1982).

In seeds of the zinc-tolerant *Silene vulgaris*, poor germination response was linked to high content of metals within the fruit/seed (Ernst and Nelissen, 2000). In a previous study on the spatial distribution and localisation of Ni within the *S. tryonii* seed (Bhatia *et al*., 2003), Ni was partitioned to the fruit wall (pericarp) while endospermic and cotyledonary tissues possessed very little Ni. However, the amount of Ni within the pericarp was insufficient to affect germinability of *S. tryonii* seeds. Indeed, exclusion of metals from embryonic tissues presumably ensures the high reproductive success of hyperaccumulating species on metal-enriched soils.

The present study investigated the effects of some dormancy-relieving treatments on seed germination of *S. tryonii* under light and dark conditions and the relationship between fatty acid content and germinability of the seeds.

MATERIALS AND METHODS

Seed material

Mature seeds of *S. tryonii* F. M. Bailey were collected in December 1996, February 2000 and August 2000. Owing to the rarity of the species, seed collection on each occasion was limited to only five plants from a single population growing naturally on ultramafic soils at Marlborough Nickel Mine lease area (22°57·36′S, 149°51·95′E). The seeds were placed in screw-capped plastic containers, wrapped in aluminium foil and stored at room temperature. This study was undertaken using seeds removed from the stored seed lots, along with seeds freshly harvested in August 2001 from plants grown on ultramafic soil in a glasshouse located within Central Queensland University. Seeds were treated with tetrazolium chloride (1 %) to determine the percentage of dormant seeds (ISTA, 1985; Tao and Fu, 1993), as dormant seeds, which fail to germinate, could result in an underestimate of seed viability. Dry weight of seeds was determined by holding them at 70 °C until constant weight was obtained.

Germination treatments

Experiment 1. Freshly harvested seed lots of *S. tryonii* and lots having different periods of after-ripening were tested for their germinability without exposing them to any dormancy-relieving treatments. Seeds were nicked, steeped in water for 6 h and placed on filter paper resting on two sheets of sponge (2·5 mm thick; moistened with distilled water) contained in deep-bottom sterile Petri plates (90 mm diameter). The sponge sheets were re-moistened with distilled water at 2-weekly intervals during the period of experimentation. Seeds were germinated in a temperature-controlled growth cabinet set at 25/20 °C (day/night cycle). Petri plates were exposed to a 12 h light/12 h dark cycle. Illumination was provided by white fluorescent tubes with mean photon flux density (400–700 nm) of 40 $\mu mol^{-2}\ m^{-1}\ s^{-1}$ at seed level.

Petri plates (with seeds) were arranged in the growth cabinet in a completely randomized design. Seed germination was assessed as radicle emergence after 6 weeks.

Experiment 2. Germinability of 4·5-year-old seed lots was tested following exposure to dormancy-relieving treatments, in the presence or absence of light. Seeds were germinated by exposing them to a 12 h light/12 h dark cycle as described above. In the dark treatment, the Petri plates were covered with two layers of aluminium foil.

Dormancy-relieving treatments

Primed control. Seeds were leached in running tap water for 36 h. Following leaching, seeds were dehydrated by spreading on a plastic tray for 20 d at room temperature (approx. 25 °C).

Non-primed control. Seeds were not leached and dehydrated prior to the germination test.

Dormancy-relieving treatments constituted exposure of leached and dehydrated seeds to the following treatments prior to the germination tests:

Treatment with gibberellic acid (GA_3). The effect of GA_3 was investigated by steeping seeds in either 150 μM or 300 μM GA_3 solutions for 16 h under ambient conditions.

Treatment with aqueous smoke extract. Seeds were steeped in either 5 or 10 % aqueous smoke extracts (Regen 2000 Smokewater™; Tecnica Pty Ltd, Bayswater, Victoria, Australia) for 16 h under ambient conditions.

Treatment with potassium cyanide solution. Seeds were steeped in either 40 or 80 mM KCN (Sigma Chemical Company, USA) solution for 30 min under ambient conditions. Thereafter, seeds were washed in running tap water for 30 min and rinsed with distilled water.

Following priming with GA_3, smoke extract or KCN, seeds were placed in Petri plates for germination tests as previously described.

Extraction and analyses of lipids

Seed tissue (200 mg) was finely ground using a pestle and mortar, and extracted for 10 min with 10 mL of chloroform/methanol (1/2, v/v) solvent system, containing 150 mM acetic acid and 0·02 % of the anti-oxidant butylated hydroxytoluene. Following centrifugation at 1500 *g*, 10 mL of chloroform was added to the supernatant, mixed and transferred to a separation funnel. The bottom or chloroformic fraction was dried under nitrogen at 60 °C

TABLE 1. *Germination responses of freshly harvested and after-ripened seed lots of* Stackhousia tryonii

Seed lot	Approximate age (years)	External colour of seeds	Moisture content (%)	Dormancy (%)	Germination (%)
Dec 1996	4·5	Dark brown	6·0 ± 0·2	72	28·3 ± 2·4
Feb 2000	1·5	Brown	5·9 ± 0·3	62	38·3 ± 4·6
Aug 2000	1	Green-yellow	6·3 ± 0·4	90	10 ± 0·0
Aug 2001	Freshly harvested	Green	7·4 ± 0·2	100	0

Moisture content and germination values shown are means ± s.e. Seeds were germinated under light [white fluorescent tubes with mean photon flux density (400–700 nm) of 40 $\mu mol^{-2}\ m^{-1}\ s^{-1}$ at seed level]. The ungerminated seeds in all seed lots were viable (stained pink with tetrazolium).

(Griffiths *et al*., 2000). Fatty acids were quantified as their fatty acid methyl ester derivatives, obtained by transmethylation with 14 % solution of BF_3 (boron trifluoride) methanol (Sigma Chemical Company, USA). The fatty acid methyl esters were analysed using a Varian 3000 (Type 3400) gas chromatograph equipped with a flame ionization detector. Separation was achieved on an Alltech Econo-Cap Carbowax column (30 m × 0·32 mm I.D.) and operated isothermally at 240 °C with nitrogen (3·2 kg cm^{-2}) as carrier gas. Fatty acids were identified by co-chromatography using methyl esters of lipid standards (Sigma Chemical Company, USA). Area percentages of fatty acids are given as the mean of three determinations.

Statistical analysis

Seed germination was analysed as a generalized linear model (GLM; McCullagh and Nelder, 1989) assuming a binomial distribution with the dispersion parameter fixed at unity and a logit link function using the GenStat statistical package (GenStat Committee, 2002). The models employed included the main effects of dormancy-relieving treatments, light and their interaction. If the interaction was not significant ($P > 0{\cdot}10$), it was removed from the model to allow testing of the main effects. Standard errors and pairwise comparisons of means were predicted based on the final model.

RESULTS AND DISCUSSION

All seed lots were viable (stained pink with tetrazolium chloride). Freshly harvested seeds of *S. tryonii* did not germinate, and development of germinability was associated with storage of the mature seeds (Table 1). Germinability increased with post-harvest storage (Table 1), up to a storage period of 18 months, beyond which it decreased. Germinability of the 4·5-year-old seeds was lower than that of the 18-months seed, but the difference was not significant ($P > 0{\cdot}05$). However, an optimum after-ripening period could not be determined on the basis of the present results. Further investigations on the effects of some dormancy-relieving treatments utilized the 4·5-year-old seeds due to a limited supply of the 1·5-year-old seeds.

As *Stackhousia tryonii* seed is tiny, germination on the soil surface would be ecologically advantageous; therefore a light response was anticipated. However, light did not affect seed germination and there was no interaction ($P > 0{\cdot}10$) between priming treatment and light (hence main effects are presented in Table 2). No significant ($P < 0{\cdot}05$) response to GA_3 (150 and 300 μM) was recorded. Since gibberellic acid can overcome the light requirement in dormant seeds (Thomas, 1992), the results suggest that the dormant condition in *S. tryonii* seed is not due to a light requirement.

Seed germination was negatively influenced ($P < 0{\cdot}05$) by priming treatments (Table 2). None of the dormancy-relieving treatments significantly improved germination. Maximum germination response was recorded with the unleached seeds, suggesting that in *S. tryonii* seed germination is not limited by the occurrence of soluble inhibitors.

Potassium cyanide, a respiratory inhibitor, has been used to stimulate germination in seeds of a variety of plant species (Esashi *et al*., 1996; Maruyama *et al*., 1996; Nkang, 1996; Keeley and Fotheringham, 1998). However, a significant reduction in the germination of seeds treated with KCN (40 and 80 mM) was noted in the present study (Table 2).

Smoke and smoke extracts are known to cue germination in species from both fire-prone and fire-free habitats (Brown and van Staden, 1997, 1998). Roche *et al*. (1997*b*) studied the effect of aerosol smoke on germination of *Stackhousia pubescens* and *Tripterococcus brunonis* (Stackhousiaceae) and noted higher germination values in aerosol-treated seed lots, but the difference between control and treated seed lots was not significant. In another study, Roche *et al*. (1997*a*) reported more than a doubling of germination and early seed emergence in *S. pubescens* compared with the control following smoking of fresh seed lots after sowing. In the present study, seed germination was significantly lower in seeds primed with smoke water. However, the lower concentrations (5 %) of the aqueous smoke extract inhibited seed germination much more than the higher concentration (10 %).

It has been suggested (Berrie *et al*., 1979) that certain short-chain (particularly C1 to C12) saturated carboxylated acids inhibit various processes in plants, including germination (Rogoyski and Powell, 1981; Bewley and Black, 1982). Freshly harvested (dormant) *S. tryonii* seeds possessed higher proportions of saturated shorter-chain fatty acids ($C_{13:0}$) relative to the total fatty acids than did after-ripened seeds (Table 3). The content of saturated fatty acids was very low or not detectable in the stored seeds. In contrast, saturated fatty acids constituted about 90 % of the total fatty acids in the oil of freshly harvested seeds. Among these, tridecanoic acid ($C_{13:0}$) was the dominant fatty acid, followed by palmitic acid ($C_{16:0}$). With post-harvest storage, there was increased accumulation of mono-unsaturated and

TABLE 2. *Effect of some priming treatments on germination of 4·5-year-old* Stackhousia tryonii *seeds*

Treatments	Germination (%)	s.e.m.
Controls		
Non-primed control*	23·3[a]	3·85
Primed control[†]	19·2[a]	3·59
Priming treatments		
L + D + GA_3 (150 μM)	17·8[a]	3·48
L + D + GA_3 (300 μM)	18·3[a]	3·52
L + D + KCN (40 mM)	8·4[c]	2·48
L + D + KCN (80 mM)	9·3[bc]	2·62
L + D + Smoke water (5 %)	6·2[c]	2·14
L + D + Smoke water (10 %)	16·5[ab]	3·38

Values represent the mean percentage germination of four individual replicates of 15 seeds each under light and dark treatments.

*Seeds were neither leached nor dehydrated.

[†]Seeds were leached (L) in running tap water for 36 h and dehydrated (D) by spreading on a plastic tray for 20 d at room temperature (approx. 25 °C).

Means followed by the same letter are not significantly different ($P < 0·05$); s.e.m., standard error of mean.

TABLE 3. *Fatty acid composition (% total fatty acids) of seed oil in freshly harvested and stored* Stackhousia tryonii *seed*

	Storage period (years)			
Fatty acid	Freshly collected	1	1·5	4·5
Saturated fatty acids				
Tridecanoic acid ($C_{13:0}$)	87·6 ± 7·5	9·5 ± 0·5	3·6 ± 0	ND
Palmitic acid ($C_{16:0}$)	5·5 ± 0·6	16·5 ± 0·8	16·2 ± 0·9	21·1 ±1·7
Stearic acid ($C_{18:0}$)	ND	1·0± 0	ND	ND
Nonadecanoic acid ($C_{19:0}$)	ND	Tr	Tr	Tr
Tricosanoic acid ($C_{23:0}$)	ND	Tr	Tr	Tr
Mono-unsaturated fatty acids				
Oleic acid ($C_{18:1}$)	ND	12·7 ± 1·1	20·3 ± 1·3	12·6 ± 0·9
Erucic acid ($C_{22:1}$)	ND	Tr	Tr	Tr
Poly-unsaturated fatty acids				
Linoleic acid ($C_{18:2}$)	7·5 ± 0	27·3 ± 1·9	34·0 ± 2·0	27·0 ± 0·9
Linolenic acid ($C_{18:3}$)	6·2 ± 0	36·8 ± 4·0	27·1 ± 1·1	39·3 ± 1·6

Seeds were collected from the same location and stored under ambient conditions in the laboratory. Fatty acid results are means of three determinations (±s.e.).

ND, not detected; Tr, traces (concentrations less than 1 % of the total fatty acids).

poly-unsaturated fatty acids, constituting about 75–80 % of the total fatty acids. Oleic acid ($C_{18:1}$) was the principal mono-unsaturated fatty acid in aged seeds. The principal poly-unsaturated fatty acids were linoleic acid ($C_{18:2}$) and linolenic acid ($C_{18:3}$) and their proportions increased more than four-fold in the aged seeds relative to contents in freshly harvested seeds (Table 3). Further studies are required for the *in vitro* evaluation of the inhibitory properties of the short-chain fatty acids on germination of *S. tryonii*.

In summary, the results of the present study suggest that poor germination response in *S. tryonii* is not due to physical dormancy or the presence of soluble inhibitors. The seeds may require an after-ripening period for germination. The poor germination potential also highlights the need to develop other propagation techniques in order to enhance the potential utilitarian benefits of *S. tryonii.* We have made some progress in establishing protocols for vegetative propagation (Bhatia *et al*., 2002*a*) and micropropagation (Bhatia *et al*., 2002*b*,*c*).

ACKNOWLEDGEMENTS

The merit scholarship (UPRA) for doctoral research to NPB by the Central Queensland University is gratefully acknowledged. We thank Queensland Parks and Wildlife Services (Central Region) for granting a scientific permit to collect *S. tryonii* material from its natural habitat, and David Reid (Senior Biometrician, DPI, Rockhampton) for assistance with the statistics.

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