

Understanding the germination of bulbils from an ecological perspective: a case study on Chinese yam (*Dioscorea polystachya*)

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Received: 6 July 2010 Returned for revision: 28 July 2010 Accepted: 5 August 2010 Published electronically: 29 September 2010

- **Background and Aims** Bulbils serve as a means of vegetative reproduction and of dispersal for many plants; this latter aspect making them analogous to seeds. However, germination of bulbils may differ considerably from seeds due to dissimilar anatomical structures and perhaps environmental cue perception. The few laboratory studies done on bulbils suggest that their germination is similar to that of seeds in the same habitats and to vegetative buds of winter-dormant plants. The present study is the first to examine how bulbil germination is controlled in nature in relation to dispersal (before vs. after winter of the same cohort) and to ambient temperatures.
- **Methods** Under laboratory conditions, temperature and light requirements for root and shoot emergences from bulbils of *Dioscorea polystachya* collected in September, 2005, February, 2006 (produced in 2005) and July, 2006 were determined. Effects of cold stratification and dry storage for releasing dormancy were tested on September and July bulbils. The phenology of dormancy release and of root and shoot emergences and the persistence of bulbils in soil were followed over time under field conditions.
- **Key Results** Although a low percentage of bulbils collected in July or in September produced roots, but no shoots, in the laboratory and field, these roots died within approx. 1 month. Regardless of collection date, cold stratification markedly increased root and shoot emergences. Bulbils sown outdoors in October produced roots and shoots the following March and April, respectively. The soil bulbil bank is short lived.
- **Conclusions** Bulbils of *D. polystachya* are similar to seeds of many temperate plants being mostly dormant when dispersed in summer or autumn and overcoming dormancy with cold stratification during winter. Adaptively, bulbil germination primarily occurs in spring at the beginning of a favourable period for survivorship and growth.

Key words: Aerial tubers, bulbils, *Dioscorea polystachya*, Dioscoreaceae, dormancy, germination ecology, soil bulbil bank.

INTRODUCTION

Bulbils often are produced in combination with seeds in plants (Ceplitis and Bengtsson, 2004) with a trade-off occurring between vegetative and sexual reproduction so that one cannot be increased without reducing the other (Law *et al.*, 1983). However, *Allium oleraceum* (Duchoslav, 2001), *Gagea spathacea* (Schnittler *et al.*, 2009), *Titanotrichum oldhamii* (Wang *et al.*, 2004) and populations of *Dioscorea polystachya* in North America (Raz, 2002) rely almost entirely on bulbils for reproduction. Bulbils may be released from a plant and grow independently or remain attached to the parent while daughter plants develop from them (Callaghan *et al.*, 1997). From a dispersal viewpoint, bulbils could be considered analogous to seeds. Bulbils are the sole means of dispersal for *D. polystachya* in North America (Thomas *et al.*, 2005), while they may disperse the same distance as seeds for *Allium vineale* (Ronsheim, 1994).

A bulbil consists of a small bud, with a short stem bearing fleshy scale leaves or leaf bases (Bell, 2008). The term also applies to axillary stem tubers, bearing one or more buds, found on the aerial stems of some climbers, which are sometimes referred to as aerial tubers. A seed is a mature ovule consisting of an embryonic plant together with a store of food

surrounded by a protective coat. Because bulbils lack the anatomical structures of seeds, it has been presumed that bulbils may not perceive environmental cues like them, as evidenced in some species by the lack of specific temperatures for germination (Dormann *et al.*, 2002). However, almost all the endodormant vegetative buds of winter-dormant temperate and subtropical plants require some sort of vernalization, which is similar to stratification in seeds, in order to sprout (Horvath *et al.*, 2003; Rohde and Bhalerao, 2007). Thus, bulbils (and their buds) could behave like seeds and use ecological cues to determine optimal times of germination and growth.

Production of bulbils occurs among plants growing in tropical forests (Wang *et al.*, 2004), temperate regions (Okagami, 1979; Ronsheim, 1994), and Arctic and alpine tundra (Wehrmeister and Bonde, 1977; Dormann *et al.*, 2002). The limited number of studies conducted on bulbil germination, all performed under laboratory conditions, suggests that bulbils from tropical plants are non-dormant and may germinate shortly after dispersal (Wang and Cronk, 2003). In contrast, bulbils from temperate species require either after-ripening to overcome dormancy with germination observed in late summer to autumn (Krochmal, 1960; Stritzke and Peters, 1970; Ronsheim, 1994) or cold stratification for dormancy break with germination presumably

occurring in spring (Krochmal, 1960; Okagami, 1979; Okagami and Tanno, 1991). Bulbils produced from plants growing in tundra are apparently non-dormant but bulbils dispersed in late summer or early autumn delay germination until the following spring (Dormann *et al.*, 2002).

The dormancy profiles of bulbils in the above studies are similar to the prevalent profiles of seeds from taxa occurring in the same vegetation types. Seeds of the majority of species in the tropics are non-dormant at maturity, whereas those of temperate species have physiological dormancy with after-ripening or cold stratification requirements (Baskin and Baskin, 1998). Moreover, seeds of many tundra species are non-dormant or physiologically dormant. Over a latitudinal gradient in Japan, Okagami (1986) found that the dormancy and germination characteristics of bulbils and seeds were similar. Therefore, bulbils apparently also are analogous to seeds in terms of their germination ecology requirements.

Laboratory studies have been conducted on bulbil germination in *Dioscorea* spp. (Okagami, 1979, 1986; Okagami and Tanno, 1991; Overholt *et al.*, 2007), *Allium* spp. (Krochmal, 1960; Stritzke and Peters, 1970), *Titanotrichum oldhamii* (Wang and Cronk, 2003) and *Polygonum viviparum* (Dormann *et al.*, 2002). However, we are unaware of any study that has related dormancy break and germination of bulbils in controlled (laboratory) conditions to the timing of these events in nature. To this end, knowledge on bulbil germination from an ecological perspective is very limited.

The genus *Dioscorea* (Dioscoreaceae), commonly known as yams, consists of about 630 species in tropical and temperate climates, many of which are important in medicine and agriculture (Mabberley, 2008). Species in this genus are well known for producing bulbils (aerial tubers) in their leaf axils (Murty and Purnima, 1983). Temperate Asian *D. bulbifera*, *D. japonica*, *D. opposita* and *D. pentaphylla* bear bulbils that are dormant at maturity and require cold stratification (Okagami, 1986; Okagami and Tanno, 1991). Endogenous phenolic compounds, called batatasins, are associated with the control of dormancy in *Dioscorea* bulbils with cold stratification lowering the amount of these chemicals (Hasegawa and Hashimoto, 1973, 1974, 1975; Hashimoto *et al.*, 1972; Ireland *et al.*, 1981). Compounds known to control dormancy in seeds also are found and are associated with dormancy in *Dioscorea* bulbils: gibberellins (Tanno *et al.*, 1992) and abscisic acid (Hashimoto *et al.*, 1968; Hasegawa and Hashimoto, 1973, 1975). In contrast to most seeds, application of gibberellic acid to bulbils generally extends dormancy (Okagami and Nagao, 1971; Hasegawa and Hashimoto, 1974; Okagami and Tanno, 1977; Wickham *et al.*, 1984). Among all groups of plants that produce bulbils, the physiological biochemistry of bulbil dormancy is best understood in *Dioscorea* (Degras, 1993; Craufurd *et al.*, 2001).

The purpose of the present study was to examine the dormancy status of bulbils from *Dioscorea polystachya* in relation to the control of germination in nature. Specifically, we determine (1) the temperature and light requirements for root and shoot emergences from bulbils collected within the growing season of formation and after over-wintering on the ground or on vines, (2) the effects of cold stratification and dry storage on dormancy release for roots and shoots, (3) the phenology of root and shoot emergences under natural conditions and (4) persistence of bulbils in soil.

MATERIALS AND METHODS

Study species

The perennial vine *Dioscorea polystachya* Turcz. (*D. batatas* Decne.; *D. opposita* auct.; *D. opposita* Thunb., an illegitimate name; *D. oppositifolia* L., misapplied and refers to an Indian species), commonly called Chinese yam or cinnamon vine, grows in temperate forests, scrub forests, herb communities, on mountain slopes, and along rivers and roadsides in China, Korea and Japan (Ting and Gilbert, 2000; GRIN, 2010). It was brought to the United States between 1910 and 1920 for ornamental use (Thomas *et al.*, 2005) and has escaped from cultivation, posing a threat to native plants from Vermont to Kansas and south to Texas and Florida (USDA, NRCS, 2010). The species is widely cultivated in Asia.

Shoots of this species in North America emerge in late April or May. Two types of tubers are produced: underground tubers up to 1 m in length that resprout annually and smaller axillary tubers (bulbils) up to 2 cm in diameter, which resemble small potatoes. The species reproduces solely by bulbils in North America (Raz, 2002), producing up to 84 bulbils m⁻¹ of stem, but by bulbils and seeds in Asia. The species is dioecious, but plants in North America are mainly staminate with only one pistillate specimen observed during a taxonomic survey (Raz, 2002). Bulbils are present on plants starting in June and increase in size while attached to the mother plants and reach approximately full size in October before being dispersed by gravity and by water in streams from late autumn to late winter (Beyerl, 2001; Raz, 2002; Thomas *et al.*, 2005, 2006; Main *et al.*, 2006).

Plant material

Bulbils of *D. polystachya* formed in summers 2005 and 2006 were collected in Rutherford County, Tennessee (USA), on 21 September 2005 and on 20 July 2006, respectively. Diameters of 25 bulbils each were measured from both of these collections. Over-wintered bulbils were collected on 28 February 2006 (produced in the 2005 growing season) from the ground surface or from vines. Experiments were started within 12 d after collections.

General laboratory procedures

Studies were performed in temperature- and light-controlled incubators set at 12/12-h daily thermoperiods of 15/6, 20/10, 25/15, 30/15 and 35/20 °C. These thermoperiods approximate mean daily maximum and minimum monthly air temperatures in Tennessee and adjacent states during March and November, 15/6; April and October, 20/10; May, 25/15; June and September, 30/15; and July and August, 35/20 °C (NOAA, 2003). The daily photoperiod was 14 h, extending from 1 h before the beginning of the high-temperature period to 1 h after the beginning of the low-temperature period. Another incubator, set at a constant 5 °C with 14-h photoperiod, was used for cold stratification. The light sources were 20-W cool white fluorescent tubes with a photon flux density (400–700 nm) at bulbil level from 48 (top shelf) to 72 (bottom shelf) mol m⁻² s⁻¹.

Bulbils were placed on white quartz sand, moistened with distilled water, in clear polyethylene containers (11.7 ×

4.3 cm) covered with plastic wrap during incubation and with polyethylene lids during stratification. Containers with bulbils incubated in darkness were additionally wrapped in aluminium foil. Water was added to the containers to keep the substrate moist. Unless otherwise stated, three replications of 25 bulbils per container were used per treatment or control.

Protrusion of the radicle or shoot (at least 2 mm long) was the criterion for root and shoot emergence, respectively; 'germination' refers to emergence of both root and shoot. Non-germinated bulbils were checked with forceps to see if they were firm, indicating that they were viable, or soft, indicating they were non-viable. The following parameters were determined: percentages of bulbils producing roots and shoots based on the number of viable bulbils and numbers of roots per bilbil. Shoots typically come out after roots, and usually there is one shoot per bilbil.

Root and shoot emergences from bulbils

Bulbils collected in September 2005 and July 2006 were incubated in light at the five thermoperiods for a total of 20 and 12 weeks, respectively, with root and shoot emergences recorded at 2-week intervals. Bulbils incubated for 2 weeks are referred to as 'fresh' and those incubated for longer periods of time served as controls for cold stratification experiments (see below). Bulbils collected in 2005 also were incubated in darkness and checked only at the end of 2 or 14 weeks.

Viability of over-wintered bulbils was recorded before incubation. Viable bulbils were incubated in light or darkness at the five thermoperiods for 2 weeks. Due to the limited number of bulbils, replications were reduced: 13 bulbils in each of three containers (ground collection) and 12 bulbils in each of two containers (vine collection).

Effects of cold stratification and dry storage on root and shoot emergences

Bulbils collected in 2005 were cold stratified for 12 or 18 weeks in light or darkness and incubated at the five thermoperiods for 2 weeks in both light regimes, and those collected in 2006 were stratified for 12 or 18 weeks in light and incubated at all thermoperiods for 12 weeks in light. Bulbils both stratified and incubated in darkness were not checked until the end of the incubation period. For the 2005 experiments, another set of containers ($n=3$) was stratified in darkness and then opened to ascertain the amount of germination that occurred in darkness during the stratification period prior to incubation in darkness.

The remaining bulbils collected in 2005 were stored dry in a closed glass jar at ambient laboratory conditions (22 °C) for two experiments: (1) after 14.5 weeks of storage bulbils were incubated in light or darkness at the five thermoperiods for 2 weeks; and (2) after 29.5 weeks of storage, roots had emerged from some of the bulbils in the jar and these were used to test the effects of cold stratification on shoot emergence only. Bulbils with roots were stratified in light for 6, 12 and 18 weeks, incubated in light at 25/15 and 35/20 °C and checked at 2-week intervals until shoots emerged on all bulbils. The control was non-stratified bulbils with roots

incubated in light at 25/15 and 35/20 °C and checked at 2-week intervals for a total of 22 weeks.

Phenology of dormancy break and root and shoot emergences

Fifty bulbils collected in 2005 were placed in each of eight fine mesh nylon bags, and on 9 October 2005 were buried 7 cm deep in plastic pots (15 cm wide \times 11 cm deep). Although we do not know how deep bulbils are normally buried in nature, we selected this depth to ensure that bulbils would not be exposed to light either by light penetration through soil or by soil disturbances from animals digging in the pots. The soil used was limestone-derived topsoil, which is typical for central Tennessee. The pots were placed outdoors, and the soil was kept continuously moist either by direct precipitation or by watering. One bag of bulbils was chosen randomly and exhumed on the 15th of each month between November 2005 and May 2006. The bag was cut open and germination parameters were recorded. In addition, non-germinated bulbils were incubated in light at 25/15 °C for 2 weeks and parameters were then recorded. The number of replicates and of bulbils per replicate varied among the exhumation dates depending on the number of bulbils available for testing.

Three replications of 100 bulbils each collected in 2005 and 2006 were sown on soil in plastic trays (16 cm wide \times 24 cm long \times 8 cm deep) on 9 October 2005 and on 22 July 2006, respectively; flats were filled with about 4 cm of soil. The flats were placed outdoors, covered with metal screen and protected from direct precipitation. At weekly intervals, all bulbils with roots and shoots were counted between sowing and the termination of the study: 4 November 2006 (2005-sown bulbils) or 25 May 2007 (2006 bulbils). To provide optimal germination conditions, soil in the flats was watered daily, except when frozen, from the time of sowing until 1 May 2006 (2005 bulbils) or 25 May 2007 (2006 bulbils). For the 2005 bulbils, from 1 May to 31 August 2006, soil was watered to field capacity once a week to simulate field conditions, and then daily watering resumed on 1 September 2006. Mean maximum and minimum daily air temperatures for each week of the study were calculated from data collected at a weather station at Murfreesboro, Tennessee, approx. 5.5 km from the study site.

Formation of a soil bilbil bank

On 12 May 2006 following the spring germination period, 20 soil samples (15 cm long \times 15 cm wide \times 7 cm deep) were examined at three population sites in Rutherford County, Tennessee: Lytle Creek Greenway (35°51'12"N, 86°24'31"W), Fortress Rosecrans (35°51'5"N, 86°24'31"W), and Artillery Monument (35°53'23"N, 86°25'30"W). The first two sites were located approx. 0.2 km apart, and both of these were approx. 4.5 km from the third site. Before sampling, *D. polystachya* at Lytle Creek Greenway was never sprayed with herbicides in a management eradication programme but it was treated at Fortress Rosecrans in 2003 and 2004 and at the Artillery Monument in 2004. Soil was sampled at 1-m intervals along transects set up in the sites. Numbers of viable, non-germinated bulbils, non-viable bulbils and juvenile plants were recorded on top of the soil surface and under the

soil surface. Juvenile plants were placed into two groups based on whether the shoot was directly attached to a bulbil (presumably <1 year old and germinated in spring 2006) or indirectly via a tuber-like structure (presumably ≥ 1 year old).

Statistical analyses

Parameters were compared by analyses of variances (ANOVAs) followed by protected least significant difference tests (SPSS, 2004). A three-way ANOVA examined the effects and interactions of condition (fresh, control, dry storage, 12-week cold stratified, 18-week cold stratified), light regime and thermoperiod for the 2005 stratification experiment and a two-way ANOVA assessed the effects and interactions of condition (0-, 12- and 18-week cold stratification) and thermoperiod at week 12 of incubation for the 2006 experiment. Percentages were arcsine square-root transformed for statistical tests. No transformations corrected the heteroscedasticity for numbers of roots per bulbil, but ANOVAs are robust against departures from variance equality especially when sample sizes are the same.

RESULTS

Requirements for root and shoot emergences from bulbils

Condition, light regime and thermoperiod significantly affected percentages of root and shoot emergences and the number of roots produced from bulbils collected in 2005 ($P < 0.0001$).

These parameters within thermoperiods and light regimes were highly dependent on bulbil condition (all two- and three-way interactions, $P < 0.0018$). Mean (\pm s.e.) diameter of these bulbils was 9.2 ± 1.3 mm. Between 0 and 9 % of non-stratified (fresh, dry-stored, control) bulbils produced roots in light or darkness over the range of thermoperiods (Table 1). Although fresh bulbils produced roots, the roots were dead by the 6th week of incubation. In contrast, 71–99 % of bulbils cold stratified for 18 weeks produced roots at 20/10–35/20 °C regardless of the light regime during stratification and incubation. The numbers of roots per bulbil increased with increasing amounts of cold stratification. Only 18-week cold-stratified bulbils produced shoots: 37, 8 and 27 % in light and 33, 55 and 55 % in darkness at 25/15, 30/15 and 35/20 °C, respectively (data not shown). Length of time for shoots to emerge from bulbils, in which roots had previously emerged during laboratory storage, decreased from 10–12 to 2 weeks with 0 and 18 weeks of stratification, respectively, irrespective of the incubation temperature (Fig. 1).

Condition and thermoperiod significantly affected percentages of root and shoot emergences from bulbils collected in 2006 ($P < 0.0001$). Emergence within thermoperiods was highly dependent on bulbil condition (two-way interaction, $P \leq 0.0114$). Mean bulbil diameter was 7.4 ± 1.1 mm. Up to 39, 96 and 100 % of control, 12-week cold-stratified and 18-week cold-stratified bulbils, respectively, produced roots in light during 12 weeks of incubation (Fig. 2). Roots on the control bulbils were dead (or turning brown) by the 6th week of incubation. The broadest range of temperatures over

TABLE 1. Root emergence (mean \pm s.e.) from non-stratified and cold-stratified bulbils of *Dioscorea polystachya* collected in September 2005

Parameter	Light regime		Incubation thermoperiod (°C)				
	Str.	Inc.	15/6	20/10	25/15	30/15	35/20
Percentage of bulbils producing roots							
Non-stratified: Fresh		L	0 ^{Aa}	1 \pm 1 ^{Aa}	0 ^{Aa}	5 \pm 3 ^{ABa}	3 \pm 3 ^{Aa}
		D	0 ^{Aa}	0 ^{Aa}	1 \pm 1 ^{Aa}	3 \pm 3 ^{ABa}	1 \pm 1 ^{Aa}
Non-stratified: Dry-stored		L	0 ^{Aa}	0 ^{Aa}	3 \pm 3 ^{Aa}	3 \pm 1 ^{ABa}	5 \pm 3 ^{Aa}
		D	0 ^{Aa}	0 ^{Aa}	1 \pm 1 ^{Ab}	9 \pm 3 ^{Bb}	7 \pm 1 ^{Ab}
Non-stratified: Control		L	0 ^{Aa}	3 \pm 1 ^{ABa}	0 ^{Aa}	7 \pm 4 ^{ABa}	3 \pm 3 ^{Aa}
		D	0 ^{Aa}	1 \pm 1 ^{Aa}	4 \pm 2 ^{Ab}	0 ^{Aa}	7 \pm 3 ^{Ab}
12-week cold-stratified	L	L	0 ^{Aa}	15 \pm 9 ^{Bb}	75 \pm 1 ^{Bc}	63 \pm 7 ^{Cc}	33 \pm 9 ^{Bc}
	D	D	0 ^{Aa}	43 \pm 5 ^{Cb}	91 \pm 7 ^{Cc}	91 \pm 4 ^{DEc}	87 \pm 5 ^{CDc}
18-week cold-stratified	L	L	0 ^{Aa}	71 \pm 7 ^{Db}	99 \pm 1 ^{Cc}	85 \pm 6 ^{Db}	72 \pm 8 ^{Cb}
	D	D	0 ^{Aa}	96 \pm 2 ^{Eb}	99 \pm 1 ^{Cb}	97 \pm 1 ^{Eb}	92 \pm 4 ^{Db}
Mean number of roots per bulbil							
Non-stratified: Fresh		L	0 ^{Aa}	0.3 \pm 0.3 ^{ABbc}	0 ^{Aa}	1.0 \pm 0.5 ^{BCc}	0.3 \pm 0.3 ^{Abc}
		D	0 ^{Aa}	0 ^{Aa}	0.3 \pm 0.3 ^{Aa}	0.3 \pm 0.3 ^{ABa}	0.3 \pm 0.3 ^{Aa}
Non-stratified: Dry-stored		L	0 ^{Aa}	0 ^{Aa}	0.3 \pm 0.3 ^{Aa}	1.0 \pm 0.6 ^{BCa}	0.8 \pm 0.4 ^{ABa}
		D	0 ^{Aa}	0 ^{Aa}	0.3 \pm 0.3 ^{Aa}	1.1 \pm 0.1 ^{BCb}	1.0 \pm 0 ^{ABCb}
Non-stratified: Control		L	0 ^{Aa}	0.7 \pm 0.3 ^{Bab}	0 ^{Aa}	1.0 \pm 0.5 ^{BCb}	0.3 \pm 0.3 ^{Aab}
		D	0 ^{Aa}	0.3 \pm 0.3 ^{ABa}	0.7 \pm 0.3 ^{Aab}	0 ^{Aa}	2.3 \pm 1.3 ^{Cb}
12-week cold-stratified	L	L	0 ^{Aa}	0 ^{Aa}	1.5 \pm 0.2 ^{Bc}	1.4 \pm 0.1 ^{CDbc}	1.2 \pm 0.1 ^{ABCb}
	D	D	0 ^{Aa}	1.2 \pm 0 ^{Bb}	2.1 \pm 0.1 ^{Bc}	2.1 \pm 0 ^{Dc}	2.1 \pm 0 ^{BCc}
18-week cold-stratified	L	L	0 ^{Aa}	1.6 \pm 0.1 ^{Bb}	3.6 \pm 0.4 ^{Cc}	2.1 \pm 0.2 ^{Db}	2.1 \pm 0.1 ^{BCb}
	D	D	0 ^{Aa}	2.7 \pm 0 ^{Cb}	3.4 \pm 0.2 ^{Cc}	3.7 \pm 0 ^{Ec}	4.4 \pm 0.2 ^{Dd}

Bulbils were incubated (Inc.) in light (L) or darkness (D) for 2 weeks (Fresh) and in light and darkness for 20 and 14 weeks, respectively (Control). Bulbils were stratified (Str.) at 5 °C for 12 or 18 weeks and incubated for 2 weeks in light or stratified and incubated in darkness. No bulbils germinated in darkness during the stratification period. Dry storage occurred in a glass jar at ambient laboratory conditions for 14.5 weeks. Three replicates of 25 bulbils each were used with 22–25 bulbils per replicate remaining viable until the end of the experiment. Values with dissimilar upper-case letters within columns or lower-case letters within rows are significantly different for each parameter (protected least-significant difference tests, $P = 0.05$).

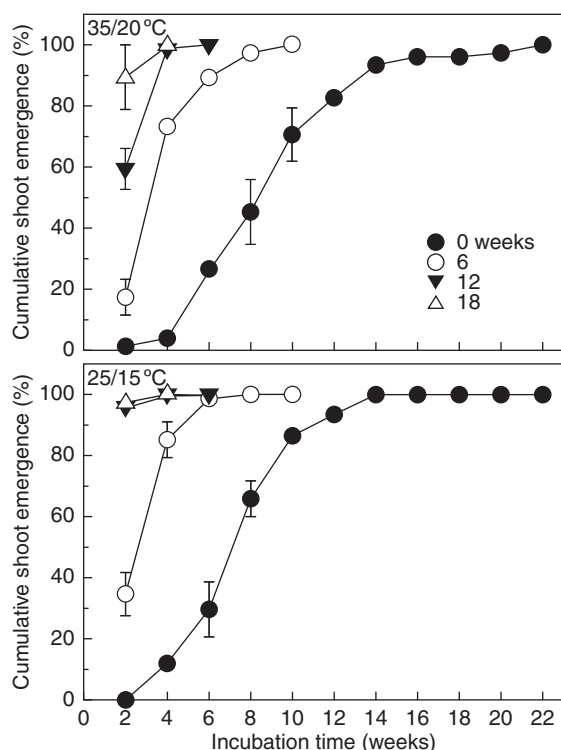


FIG. 1. Percentages (mean \pm s.e.; s.e. shown if $\geq 5\%$) of *Dioscorea polystachya* bulbils that produced shoots at two thermoperiods following 0 (control) to 18 weeks of stratification at 5°C . Roots had already emerged from these 2005-collected bulbils during storage in a glass jar at ambient laboratory conditions. Three replicates of 25 bulbils each were used at the start of the experiment and all of them remained viable until the end of it.

which high percentages of roots and shoots emerged was for bulbils cold stratified for 18 weeks.

Approximately 3% of the 733 bulbils collected in February 2006 from the ground were non-viable, whereas 40% of the 759 bulbils obtained from vines were non-viable. Between 47 and 95% of viable bulbils collected from the ground or vine produced roots when incubated in light at $20/10$ – $35/20^\circ\text{C}$, and 14–86% produced roots in darkness (Table 2). Shoots were produced from 22–64% of bulbils collected from the ground and incubated in light or darkness at $25/15$ – $35/20^\circ\text{C}$, but only 0–17% of those collected from vines.

Phenology of dormancy break and root and shoot emergences

For non-germinated bulbils exhumed from bags and incubated at $25/15^\circ\text{C}$, the percentage of root emergence and number of roots per bulbil gradually increased between November 2005 and March 2006 and the percentage of shoot emergence increased between January and March 2006 (Fig. 3). During burial in soil, only 0–2% of bulbils had produced roots between November 2005 and February, 2006 but 86–94% had done so between April and May 2006. Number of roots per bulbil increased dramatically starting in March, 2006. Shoots were present on bulbils exhumed in April and May 2006. Viability of bulbils during burial was 86–100% throughout the study.

Roots from bulbils sown on top of soil on 9 October 2005 primarily began to emerge between 12 and 18 March 2006, when mean weekly maximum and minimum temperatures were 21.3 and 11.3°C , respectively, and peak root emergence occurred between 26 March and 1 April 2006, when temperatures were 17.1 and 1.7°C , respectively (Fig. 4). Shoots began to emerge between 9 and 15 April 2006, when mean weekly maximum and minimum temperatures were 23.4 and 9.0°C , respectively, and peak shoot emergence occurred the following week (16–22 April), when temperatures were 28.8 and 16.0°C , respectively. Although germination mostly ended in May 2006, 2% of bulbils produced roots and shoots in summer 2006 but no additional bulbils did so after 15 September 2006. Of 300 bulbils sown, $19 \pm 1\%$ died during the study.

Up to 25% of bulbils sown on 22 July 2006 produced roots during the first month after sowing (until 19 August 2006), when mean weekly maximum and minimum temperatures were 32.3 and 21.1°C , respectively (data not shown). By 30 September 2006, roots on all bulbils were dead. Of the bulbils that produced roots during summer 2006, 10% of them did so again during spring 2007 with 8% bearing shoots, and of the bulbils that did not have roots during summer 2006, 23% produced roots and shoots in spring 2007. Roots started to emerge from both groups of bulbils between 17 and 23 March 2007, when mean weekly maximum and minimum temperatures were 20.0 and 4.7°C , respectively, and shoots did so between 24 and 30 March 2007, when temperatures were 28.2 and 11.5°C , respectively. No additional roots or shoots emerged after 25 May 2007, when 60% of the remaining bulbils were dead.

Formation of a soil bulbil bank

No non-germinated, viable bulbils were found on or under the soil surface in the three population sites (Table 3). Among the sites, the number of juvenile plants varied considerably between the two age classes with highest numbers of both classes at the unmanaged site (Lytle Creek Greenway).

DISCUSSION

Low percentages of fresh, dry-stored and control bulbils of *D. polystachya* collected in central Tennessee produced roots, and only over a limited range of thermoperiods (Table 1, Fig. 2). Moreover, none of these non-stratified bulbils produced shoots. However, cold stratification either in the laboratory (Table 1, Figs 1 and 2) or in the field (Table 2) dramatically altered the germination responses. Stratification increased the percentages of root and shoot emergences and the numbers of roots per bulbil and it broadened the temperature range for root and shoot emergences. Thus, we conclude that *D. polystachya* bulbils were mostly dormant in July and September and required cold stratification to become non-dormant. These results are consistent with studies on this species in northern and eastern Honshu, Japan, which have concentrated on sprouting: cold stratification effectively overcomes dormancy (Hasegawa and Hashimoto, 1973, 1975; Okagami, 1979; Okagami and Tanno, 1991). However, specific requirements may differ between the countries. For example, 0–3%

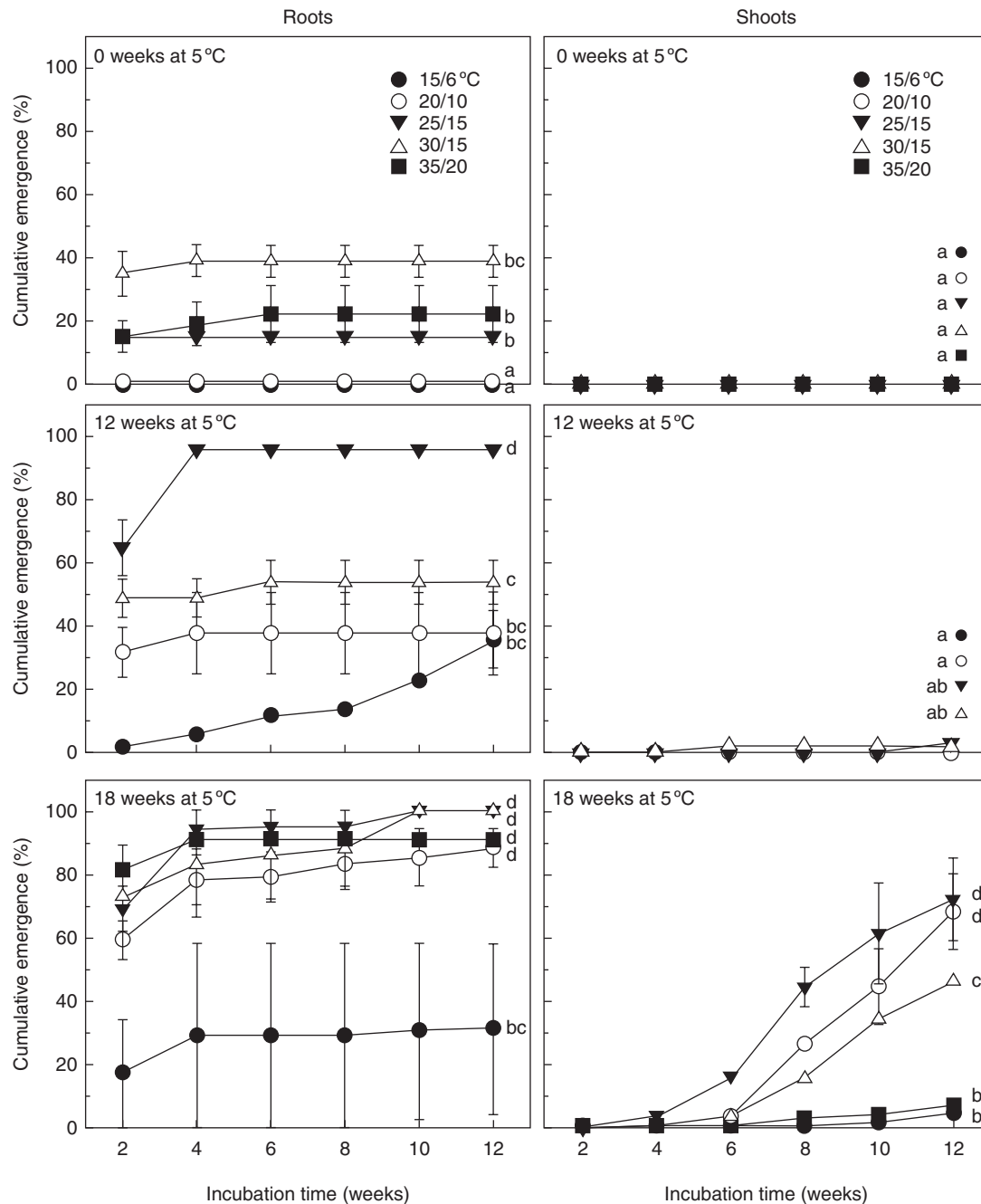


FIG. 2. Percentages (mean \pm s.e.; s.e. shown if $\geq 5\%$) of *Dioscorea polystachya* bulbils collected in July 2006 that produced roots or shoots during 12 weeks of incubation over a range of thermoperiods following 0–18 weeks of stratification at 5 °C. Three replicates of 25 bulbils each were used at the start of the experiment with 17–25 bulbils per replicate remaining viable until the end of it, with the exception of the 12 weeks at 5 °C treatment in which all bulbils at 35/20 °C died. Values among roots or shoots at week 12 with different letters are significantly different (protected least-significant difference tests, $P = 0.05$). Where data points for shoots overlap, symbols following the letters specify the associated thermoperiod.

of our bulbils bore shoots during 12 weeks of incubation following 12 weeks of cold stratification (Fig. 2) whereas 100 % of bulbils from Japan did so during 11–14 weeks of incubation after 13–14 weeks of stratification (Hasegawa and Hashimoto, 1973; Okagami and Tanno, 1991). These differences may be due to experimental conditions (e.g. moisture, temperature or light regimes), to maturity and degrees of dormancy for the

bulbils, and/or may reflect adaptations to local climatic conditions.

Bulbils kept in a glass jar also produced roots and shoots: 56 % of them had an average of 1.8 roots after 24 weeks of storage and 43 % had a small (approx. 1–2 mm long) shoot after 43 weeks (J. L. Walck *et al.*, unpubl. data). Sprouting under dry conditions on laboratory shelves has been observed

TABLE 2. Percentages (mean \pm s.e.) of bulbils producing roots and shoots from *Dioscorea polystachya* formed during the 2005 growing season and collected in February 2006 either from the ground or from vines

		Incubation thermoperiod (°C)				
Source	Light regime	15/6	20/10	25/15	30/15	35/20
Ground						
Roots	Light	0	68 ± 1	87 ± 9	72 ± 10	59 ± 9
	Dark	3 ± 3	36 ± 12	80 ± 3	47 ± 20	14 ± 2
Shoots	Light	0	0	22 ± 18	22 ± 4	48 ± 2
	Dark	0	6 ± 6	64 ± 12	24 ± 20	49 ± 20
Vine						
Roots	Light	0	72 ± 1	95 ± 4	73 ± 0	47 ± 16
	Dark	0	35 ± 8	32 ± 1	86 ± 11	67 ± 3
Shoots	Light	0	0	12 ± 1	0	0
	Dark	0	0	5 ± 4	17 ± 6	6 ± 5

Bulbils were incubated over the range of thermoperiods in light or darkness for 2 weeks. Three replicates of 13 ground bulbils and 12 vine bulbils each were used at the start of treatments with 7–13 ground bulbils and 4–12 vine bulbils per replicate remaining viable until the end of 2 weeks.

for *D. bulbifera* bulbils (Wickham *et al.*, 1982). Ethylene precursors break dormancy in *D. alata* and *D. bulbifera* bulbils: rates and percentages of sprouting increased when they were immersed in 2-chloroethanol, used in the synthesis of ethylene oxide, and ethanol, an effective substitute for 2-chloroethanol (Passam *et al.*, 1982). By contrast, Okagami (1979) showed that as oxygen concentration decreased, sprouting of immature bulbils increased but that of mature bulbils decreased. Thus, ethylene production and/or oxygen concentration in the glass jar of our study may have alleviated some dormancy in the bulbils.

In nature, a low percentage of *D. polystachya* bulbils, if dispersed between July and September, could produce roots because temperature requirements for root emergence in laboratory experiments (25/15–35/20 $^{\circ}$ C) approximate field temperatures (Table 1, Fig. 2). By contrast, we found that root emergence decreased when bulbils were collected from mid-to late summer (J. L. Walck *et al.*, unpubl. data). Indeed, root emergence was lower in our September collection (Table 1) as compared with the July collection (Fig. 2), albeit that they came from different years. In one of four Tennessee populations surveyed, we observed many bulbils with roots (up to 4 mm long) while on the vine in early to mid-August 2006 but by early September 2006 most of the roots were dead. Bulbils sown outdoors (under optimal moisture conditions) in July produced roots immediately but all roots died within a few weeks. Thus, although some bulbils may produce roots following summer dispersal, we would not expect any plant establishment from them. Starting in October and lasting throughout the rest of autumn, temperatures ($\leq 20/10$ $^{\circ}$ C) would be too low for root emergence. As such, bulbils we sowed in October never produced roots in autumn (Fig. 4)

For Japanese bulbils collected in August and grouped into size classes, those 2–4 mm in diameter sprouted without cold stratification whereas those ≥ 4.5 mm required it

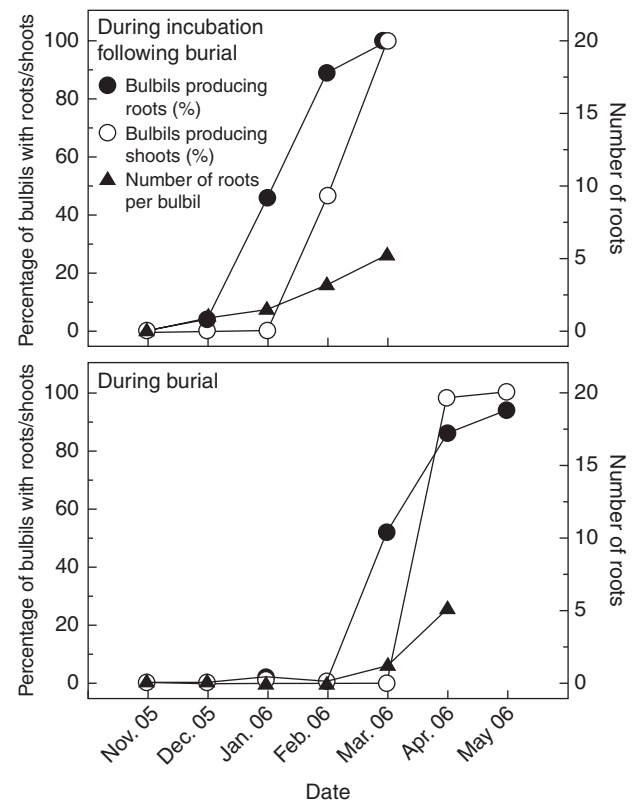


FIG. 3. Percentages of 2005-collected *Dioscorea polystachya* bulbils producing roots and shoots and the numbers of roots per bulbil during 2 weeks of incubation in light at 25/15 $^{\circ}$ C following burial or during burial between November 2005 and May 2006. No bulbils were incubated in the April and May 2006 exhumations because nearly all of them had germinated in the bags during burial; the number of roots was not recorded in the May 2006 exhumation due to difficulty in removing bulbils from bags without breaking roots. Fifty bulbils were placed into each bag; during incubation the number of replicates (three between November and January, two for February, one for March) and of bulbils per replicate (14–19 remained viable among three replications, 22–24 for two replications and 46 for one replication) varied among the exhumation dates depending on the number of bulbils available for testing. Values are means; all standard errors are $< 15\%$ or 0.6. The March 2006 data points for percentages of bulbils producing roots and shoots during incubation overlap, as indicated by the mixed black and white shaded symbol.

(Hasegawa and Hashimoto, 1973; Okagami and Tanno, 1991). Approximately 40–90 % of bulbils collected between August and early September (3–4 mm in diameter) sprouted during 24 weeks of incubation but those collected between mid- and late September (≥ 6 mm) needed cold stratification (Okagami, 1979). Neither our July collection nor our September collection produced shoots without cold stratification, regardless of bulbil diameter. Given that small (≤ 4 mm wide) bulbils in Japanese populations sprouted without cold stratification, bulbils smaller than those we tested possibly could germinate in nature. However, these small bulbils were incubated under moist conditions for 40–170 d in light at 23 or 26 $^{\circ}$ C (Hasegawa and Hashimoto, 1973; Okagami, 1979; Okagami and Tanno, 1991). This exceptional environment would not be sustained for a long length of time in nature, especially due to commonly occurring droughts in summer and autumn in central Tennessee. Although sprouting has

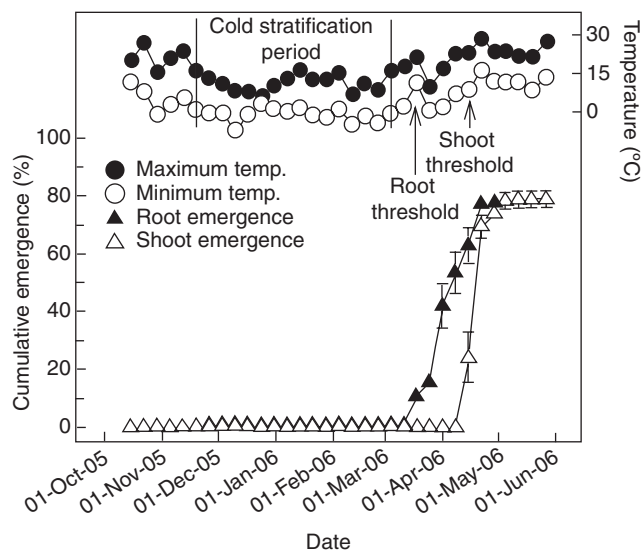


FIG. 4. Percentages (mean \pm s.e.; s.e. shown if $\geq 5\%$) of *Dioscorea polystachya* bulbils that produced roots and shoots. Bulbils were collected on 21 September 2005 and sown on soil on 9 October 2005 in flats placed outdoors. Mean weekly maximum and minimum temperatures are shown from 1 October 2005 to 27 May 2006. Cold stratification (i.e. when maximum and minimum temperatures averaged between 0 and 10 °C) is indicated between the two lines. Arrows show when the lower temperature thresholds for root and shoot emergence were reached. Three replicates of 100 bulbils each were used at the start of the study with 80–84 bulbils per replicate remaining viable until May 2006.

TABLE 3. Number (mean \pm s.e.) of non-germinated (viable and non-viable) bulbils and juvenile plants of *Dioscorea polystachya* on versus under the soil surface at three population sites in central Tennessee during May 2006 following the spring germination season

Site name and sample location	Non-germinated bulbils (m ⁻²)		Juvenile plants (m ⁻²)	
	Viable	Non-viable	<1 year old	≥ 1 year old
Lytle Creek Greenway				
On soil surface	0	4 \pm 4	720 \pm 145	93 \pm 25
Under soil surface	0	0	13 \pm 7	0
Fortress Rosecrans				
On soil surface	0	0	7 \pm 4	58 \pm 12
Under soil surface	0	0	9 \pm 7	0
Artillery Monument				
On soil surface	0	0	60 \pm 22	53 \pm 9
Under soil surface	0	0	0	0

Juvenile plants were placed into one of two groups based on whether the shoot was directly attached to a bulbil (presumably <1 year old and germinated in spring 2006) or indirectly via a tuber-like structure (presumably ≥ 1 year old).

been seen in 2-week-old bulbils presumably in the USA (Shimp, 2000; Tu, 2002), no information was provided on plant establishment from them.

Following the cold stratification period in winter, bulbils produced roots and shoots in March and April when temperatures were approximately $\geq 20/10$ and $\geq 25/15$ °C, respectively

(Tables 1 and 2, Figs 1–4). Shifts in the optimal temperature for root emergence occurred with cold stratification: 30/15 °C without stratification to 25/15 °C with 12 weeks of stratification to both temperatures being optimal with 18 weeks of stratification (Fig. 2). In addition, shoot emergence also took place at relatively high temperatures. Although we have no explanation as to the cause of the shift in optimal temperatures for roots, emergence for both roots and shoots would be delayed relative to some other species in the spring until ambient temperatures reach this high. For example, seeds of *Solidago altissima*, a forb that grows in the vicinity of *D. polystachya*, germinates to high percentages at 15/6 °C following cold stratification, and thus seedlings emerge in February (Walck et al., 1997).

Bulbils placed in bags and buried in soil in October 2005 did not have roots or shoots until they were exhumed in March 2006 and April 2006, respectively (Fig. 3). Dormancy was broken for these bulbils 2 months prior to when roots and shoots were observed on them during exhumation: non-germinated bulbils exhumed in January 2006 produced roots when incubated at 25/15 °C and in February 2006 produced shoots. Although dormancy is broken by January or February, bulbils are prevented from germinating in nature due to low temperatures. For bulbils that over-wintered on vines or on the ground, root production was high and shoot production was low (from vines) to moderate (from the ground) when they were tested for germination in February (Table 2). As such, root and shoot emergence from bulbils sown on top of soil in October 2005 started in mid-March 2006 and early April 2006, respectively, and largely ceased after May 2006 (Fig. 4). In Illinois (USA), germination of this species begins in early to mid-April and continues to November (Shimp, 2000; Thomas et al., 2006). Temperature per se was probably the primary factor controlling germination. Although the photoperiod in our incubators (14 h d⁻¹) was longer than that received under natural conditions between September and March (approx. 9–12 h d⁻¹), the long photoperiod probably had little impact given that no major differences occurred between the light and dark treatments (Tables 1 and 2).

Up to 80–95 % of bulbils collected from the ground and from vines in February produced roots in light or darkness. In contrast, only up to 64 % of ground bulbils and 17 % of vine bulbils produced shoots (Table 2). Furthermore, a higher percentage of vine bulbils were non-viable than ground bulbils when collected in February. We suggest that moisture conditions of ground vs. vine bulbils may explain the discrepancies between shoot emergence and viability like that found in seeds of *Lomatium dissectum*. In this species, interrupting cold stratification by 8 weeks of dehydration decreased seed viability and seed germination (Scholten et al., 2009). Apparently, ground and vine bulbils of *D. polystachya* had adequate moisture for 12 weeks for effective cold stratification to overcome root dormancy. In contrast, ground bulbils may have been in a more suitable moisture state than vine bulbils over an 18-week period for effective dormancy break for shoots and this moisture state may also have influenced viability.

High percentages of non-dormant bulbils produced roots and moderate to high percentages shoots in darkness under laboratory conditions (Tables 1 and 2). Likewise, cold-stratified

bulbils from Japanese populations sprouted in both light and darkness (Hasegawa and Hashimoto, 1973; Okagami, 1979; Okagami and Tanno, 1991). Nearly all bulbils in our study produced roots and shoots while buried in soil (Fig. 3). We did not find any viable (non-germinated) bulbils either on or under the soil surface in three populations sampled in May, past the peak period of germination (Table 3). Our study supports Beyerl (2001) in that *D. polystachya* bulbils germinate primarily during the first spring following dispersal and no long-term soil bulbil bank is formed. In contrast, Thomas *et al.* (2006) found that bulbils in Illinois remained viable for more than 1 year especially under 2 cm depth of leaves.

The number of juvenile plants, particularly <1 year old, varied among the study sites (highest to lowest): Lytle Creek > Artillery Monument > Fortress Rosecrans (Table 3). This hierarchy was related to the number of years that the sites had been managed before sampling occurred: Lytle Creek, never; Artillery Monument, 1 year; and Fortress Rosecrans, 2 years. Thus, herbicidal treatment was very effective in reducing bulbil production, germination and recruitment. Once bulbil production ceases at a site due to management, populations should not recover from a long-term (persistent) soil bulbil bank given its absence. However, our results do show that management over several years is needed to completely eradicate the species from these sites.

The native *D. villosa* grows throughout eastern North America, where it produces seeds and no bulbils. *Dioscorea villosa* seeds are similar to *D. polystachya* bulbils in that cold stratification is required for dormancy break, dry storage is ineffective in overcoming dormancy, germination occurs in spring following autumn dispersal, and persistence in soil is limited (Albrecht and McCarthy, 2006). Seeds of other temperate species of *Dioscorea* also require cold stratification: the eastern North American native *D. quaternata*, one species in Albania and Montenegro, one species in the Caucasian region, and nine species (including *D. opposita*) in eastern Asia (Yakuwa *et al.*, 1981; Okagami and Kawai, 1982; Okagami, 1986; Terui and Okagami, 1993). By contrast, two important differences in germination occur between *D. polystachya* bulbils and *Dioscorea* seeds. First, germination occurs at lower temperatures in seeds as compared with bulbils following cold stratification: 15/6 °C in *D. villosa* (Albrecht and McCarthy, 2006) and 8–14 °C in *D. quaternata* and all species studied in Europe and Asia (Terui and Okagami, 1993). Secondly, non-stratified seeds of some Asian species germinated to high percentages either at low temperatures (11–17 °C) and/or high temperatures (20–32 °C), but not those of North American or European species (Okagami, 1986; Terui and Okagami, 1993; Kanazawa *et al.*, 2002). Although moderate to high seed germination occurs in darkness (Yakuwa *et al.*, 1981; Kanazawa *et al.*, 2002), a comparison between light and dark regimes has not been done.

In the present study, we have shown that bulbils of *D. polystachya* need specific conditions for germination. The germination characteristics of these bulbils are similar to those of seeds from some other temperate species that co-occur with *D. polystachya* in central Tennessee, e.g. the native vines *Campsis radicans* (Bonner, 2008) and *Clematis virginiana* (Zasada and Rudolf, 2008) and the exotic vine *Lonicera japonica* (Hidayati *et al.*, 2000). Bulbils of *D. polystachya* and

seeds of the three vines require winter for dormancy release and then germinate in spring. If a bulbil or the seeds germinated in autumn, the young plants may not have time to reach a sufficient size to persist through winter and would probably die. Thus, bulbils of *D. polystachya*, like seeds of some other temperate plants, germinate at the beginning of a favourable period for establishment, at least with regard to temperature conditions.

Several caveats need to be considered regarding our study. First, although our laboratory and field studies were simulated as close as possible to natural conditions, there are obvious differences that may affect germination outcomes. For example, temperature and photoperiod variation in nature is never matched perfectly in an incubator and laboratory experiments and only represents a ‘snap-shot’ of the species’ biology. Secondly, different sizes of bulbils probably have dissimilar resource status, which may lead to different germination success. For example, survivorship of 2005 bulbils in the germination phenology study was higher than that of the slightly smaller 2006 bulbils. Thirdly, developmental effects on germination cannot be separated from year effects. During 2 weeks of incubation, nearly all shoots from the 2005 collection emerged following burial over-winter (Fig. 3) whereas none from the 2006 collection did so at the same incubation temperature (25/15 °C) following 18 weeks of cold stratification (Fig. 2). Only studies of bulbils collected over time within growing seasons during a multi-year investigation will begin to unravel the causes of these disparate results.

Unfortunately, neither the genetic diversity nor the colonization history of *D. polystachya* in North America has been studied. Whether our results can be generalized for the species on this continent or are only valid for the studied clone(s) are unknown. Differences between our work and that of other researchers (e.g. shoot emergence in Tennessee vs. Japan or differences in field emergence within North America) might be due to locally adapted clones. However, given that germination requirements of bulbils in Tennessee were similar, at least in terms of requiring cold stratification, to those in Asia and that comparable winter environmental conditions occur throughout the range of the species on both continents, we would not expect the germination ecology of other clone(s) to be too dissimilar.

ACKNOWLEDGEMENTS

This work was partially supported by the National Park Service’s Challenge Cost Share Program. The views expressed herein are those of the authors and do not necessarily represent the position of the US Department of the Interior. We thank the Stones River National Battlefield (T. Hogan, K. Hurt) and M. Rolig for their assistance with the project.

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