

# Longevity of the diaspore bank of the episodic moss *Physcomitrium eurystomum* Sendtn.

Des A. Callaghan<sup>a</sup>, Heinjo During<sup>b</sup>, Rafael Medina<sup>c</sup> and Handong Yang<sup>d</sup>

<sup>a</sup>Bryophyte Surveys Ltd, Almondsbury, UK; <sup>b</sup>Utrecht University, Utrecht, The Netherlands,

<sup>c</sup>Complutense University of Madrid, Madrid, Spain; <sup>d</sup>Environmental Change Research Centre, University College London, London, UK.

Correspondence to: Des Callaghan, Bryophyte Surveys Ltd, 10 The Scop, Almondsbury, BS32 4DU, UK. E-mail: des.callaghan@outlook.com

## Abstract

Introduction: *Physcomitrium eurystomum* is rare in Europe and threatened with extinction. This study investigates the longevity of its diaspore bank at Langmere, Norfolk, UK.

Methods:

Key results and conclusions:

Keywords: Funariaceae, lead-210, DNA barcoding, sediment core, germination trial.

## Introduction

[Introductory paragraph on diaspore banks to add]

*Physcomitrium eurystomum* Sendtn. has a broad geographic range, including parts of Africa, Asia, Australasia and Europe. Whilst it is widespread in Europe, it is generally rare and is undergoing a decline, and as such it is included as 'Vulnerable' on the IUCN Red List of bryophytes (Hodgetts et al. 2019). It is also rare and undergoing a decline in Britain, and is categorised as 'Endangered' on the national IUCN Red List (Callaghan submitted), being confined to SE England, where only five sites were found to be occupied during a recent national survey (Callaghan et al. 2020). The diaspore bank appears to be a fundamental aspect of the ecology of this species, but is virtually unstudied. It is an episodic species, typically occupying the summer draw-down zone of exposed mud around freshwater wetlands, completing its full life-cycle rapidly during the summer and autumn period, before water levels rise and inundate its habitat for the winter period. The aim of this study is to investigate the diaspore bank of *P. eurystomum*, in its potential longevity in natural habitat.

## Methods

## Study site

Langmere (52°27'40"N, 0°48'19"E), Norfolk, UK, comprises a natural freshwater wetland of three small basins, with water supplied to them by rainfall and from a groundwater aquifer located in the underlying chalk. In response to seasonal changes in the aquifer, the water level of each basin fluctuates substantially. The periodic inundation zone of the basins support a number of rare and unusual plants, including *P. eurystomum*. The moss was discovered here, new for Britain, in 1961 by B.F.T. Ducker (Ducker and Warburg 1961). The site is owned and managed as a nature reserve by Norfolk Wildlife Trust and became legally protected for its nature conservation in interest in 1954, via its inclusion within the East Wretham Heath Site of Special Scientific Interest.

## Sampling of sediment layers

In October 2019, when all three basins of Langmere were dry, three soil cores immediately adjacent to each other were extracted from the centre of the main basin, using an Eijkelcamp 04.17.01.C split tube sampler (Eijkelcamp, Giesbeek, The Netherlands). Each contained a continuous and undisturbed soil core of 5 cm diameter, ranging from the soil surface to a depth of 40 cm. At the time of extraction from the ground, each core was dry throughout its length. On the following day, each core was split into 40 soil samples, each sample comprising a 1 cm depth sediment layer of 19.6 cm<sup>3</sup>. For one of the cores, 10 cm<sup>3</sup> of soil was extracted from each sample and was sent to University College London (UCL) for dating (see below). All remaining samples were sent to the University of Utrecht for germination trials (see below).

## Dating of sediment layers

Dating of sediment layers was undertaken at the Environmental Radiometric Facility at UCL by an analysis of radionuclides, including Americium-241 (<sup>241</sup>Am; half-life = 470 yr), Cesium-137 (<sup>137</sup>Cs; half-life = 30 yr), Lead-210 (<sup>210</sup>Pb; half-life = 22.3 yr) and Radium-226 (<sup>226</sup>Ra; half-life = 1600 yr). Analysis was by direct gamma assay, using an ORTEC HPGe GWL series well-type coaxial low background intrinsic germanium detector. Lead-210 was determined via its gamma emissions at 46.5keV, and <sup>226</sup>Ra by the 295keV and 352keV gamma rays emitted by its daughter isotope <sup>214</sup>Pb following three weeks of storage in sealed containers to allow radioactive equilibration. Cesium-137 and <sup>241</sup>Am were measured by their emissions at 662keV and 59.5keV, respectively (Appleby et al. 1986). The absolute efficiencies of the detector were determined using calibrated sources and sediment samples of known activity. Corrections were made for the effect of self-absorption of low energy gamma rays within the sample (Appleby et al. 1992).

## Germination from sediment layers

Diaspore germination trials were undertaken in a growth chamber at Utrecht University (The Netherlands). Each soil sample was spread over sterilised sand in 8 x 8 cm transparent plastic containers with lids, and sprayed with demineralized water, as necessary, to maintain a moist soil surface. Light was provided at 200  $\mu\text{mol m}^{-2} \text{s}^{-1}$  (16 h light, 8 h darkness) and temperature kept at 20-22°C. The trial lasted for 628 days (29 November 2019 – 18 August 2021). Vascular plants were removed from germination boxes as they arose, to avoid competition.

## DNA barcoding

*Physcomitrium eurystomum* can only be identified with certainty when mature sporophytes are present. To help confirm the identity of plants that did not produce sporophytes during the growth trial, DNA barcoding of samples was undertaken, and the results compared with voucher specimens of *P. eurystomum* and from other Funariaceae previously sequenced.. DNA was extracted with the commercial kit NucleoSpin Plant II Mini (Macherey-Nagel, Düren, Germany) following the instructions provided by the manufacturer (PL1 as lysis buffer). Considering the hybrid origin of the species, different *loci* were targeted in both the plastid and nuclear genomes by PCR. Within the plastid genome, two *loci* were targeted: *psbA-trnH* and *trnL-F*, while within the nuclear compartment, we sequenced two flanking regions associated to the genes 4780 and 7379 used in the Targeted Enrichment capture approach described in Medina et al. (2019). The primers used for the nuclear *loci* were newly designed by Matt Johnson and Nikisha Patel: 4780F = ATGGACGGCGCACTTGTTA; 4780R = CTTGTAACGTCGCTTCAGATTTT; 7379F = TCACGTTGGACCATGTGACG; and 7379R = CGTTCAAACGCCTCTCATTG.

Polymerase Chain Reactions (PCRs) were conducted in final volumes of 50  $\mu\text{L}$ , with 0.15  $\mu\text{L}$  of GoTaq DNA polymerase (Promega, Madison WI, USA), 1  $\mu\text{L}$  of 10  $\mu\text{M}$  dNTP mix, 1  $\mu\text{L}$  of each primer (10  $\mu\text{M}$ ) and 1  $\mu\text{L}$  of DNA extract. The thermal cycler was programmed with a hot start denaturation step of 5 min at 94°C, followed by 30 cycles of denaturation (1 min, 94°C), annealing (1 min, 50°C) and extension (1 min, 70°C), ended by a final extension step of 10 min. Amplification products were visualized in 1% agarose gels, cleaned used the ExoSAP-IT protocol (USB-Affymetrix, Cleveland OH, USA), and sent for sequencing to Macrogen through the EZ-sequencing service. Contigs were generated using Geneious (Kearse et al. 2012; <http://www.geneious.com>), keeping track of all double peaks shown in both reads of each sequence of the nuclear *loci* using the standard IUPAC ambiguous

nucleotide letters in the consensus sequence, and then compared visually on PhyDe (Müller et al. 2006; <http://www.phyde.de>).

## Results

### Dating of sediment layers

Total  $^{210}\text{Pb}$  activity reaches equilibrium depth with supported  $^{210}\text{Pb}$  activity at a depth of ca. 12 cm (Table 1; Figure 1a). Unsupported  $^{210}\text{Pb}$  activities, calculated by subtracting  $^{226}\text{Ra}$  activity (as supported  $^{210}\text{Pb}$ ) from total  $^{210}\text{Pb}$  activity, decline overall irregularly with depth (Table 1; Figure 1b). The maximum unsupported  $^{210}\text{Pb}$  activity is beneath the surface sediments, suggesting an increase in sedimentation rates in recent years. Unsupported  $^{210}\text{Pb}$  activities from 1.5 to 3.5 cm decline more or less exponentially with depth, implying relatively stable sedimentation rates. A dip in unsupported  $^{210}\text{Pb}$  activity at 5.5 cm (Table 1) suggests an increased sedimentation rate at that depth. From 6.5 to 11.5 cm unsupported  $^{210}\text{Pb}$  activities show little net decline with depth, suggesting an increase trend in sedimentation rates from deep to shallow sediments.

The  $^{137}\text{Cs}$  activity versus depth profile (Figure 1c; Table 2) shows similar values between 3.5 and 6.5 cm and a peak between 1.5 and 2.5 cm, which might be derived from the fallout of the atmospheric testing of nuclear weapons with the maximum level in 1963 and the 1986 Chernobyl accident fallout, respectively. Low  $^{241}\text{Am}$  activities were detected in disconnected samples, which are not sufficient for dating (Table 2).

Use of the CIC (constant initial concentration) model was precluded by the non-monotonic variation in unsupported  $^{210}\text{Pb}$  activities.  $^{210}\text{Pb}$  chronologies were calculated using the CRS (constant rate of  $^{210}\text{Pb}$  supply) dating model (Appleby 2001). The CRS model places 1963 and 1986 between 4.5 and 5.5 cm and between 1.5 and 2.5 cm, respectively, suggesting that Langmere was affected by the 1986 fallout from the Chernobyl accident. The CRS dating model suggests that since the 1890s, sedimentation rates increased significantly during the 1930s and 1950s, but have otherwise remained relatively stable (Figure 2; Table 1).

### DNA barcoding

[Rafa to add text]

### Germination from sediment layers

Frequent and rapid germination of *P. eurystomum* occurred in many of the soil samples, and subsequent production of sporophytes occurred commonly (Figure 3). Figure 4 shows the germination of shoots of the species from the different sediment layers of the three soil cores. Results indicate that viable spores of *P. eurystomum* at Langmere are highly concentrated in the upper 20 cm of soil, but also occur lower down, including at the maximum depth sampled (i.e. 39–40 cm). When considering the sediment layer at 10–11 cm depth is 121 ( $\pm 26$ ) years old, the results suggest that spores of *P. eurystomum* retain high viability for over a century, and that some spores may remain viable for several centuries.

## **Discussion**

[To be completed]

## **Acknowledgements**

Many thanks to the following for various kind help and support: John Birks (University of Bergen), Emily Dimsey (Norfolk Wildlife Trust), Ash Murray (Norfolk Wildlife Trust), Jonathan Preston (Norfolk Wildlife Trust) and Neil Rose (University College London).

## **Funding**

Funding for the sediment dating was provided by The British Bryological Society.

## **Notes on contributors**

Des Callaghan is a consultant bryologist operating under Bryophyte Surveys Ltd, working throughout Britain and further afield. His research is focused on threatened species, taxonomy and conservation ecology.

Heinjo During is a retired plant ecologist and bryologist of the Ecology and Biodiversity Group of Utrecht University.

Rafael Medina is assistant professor in the Department of Biology of Augustana College (Illinois, USA). His main research interest is bryophyte systematics.

Handong Yang is a Principal Research Fellow working at University College London. His main research interest is environmental pollution and sediment dating....

## **ORCID**

Des A. Callaghan <http://orcid.org/0000-0002-0415-1493>

## References

- Appleby PG. 2001. Chronostratigraphic techniques in recent sediments. In: Last WM, Smol JP. eds. Tracking environmental change using lake sediments. Volume 1. Basin analysis, coring and chronological techniques. Dordrecht: Kluwer Academic Publishers, pp 171–203.
- Appleby PG, Richardson N, Nolan PJ. 1992. Self-absorption corrections for well-type germanium detectors. Nuclear Instruments and Methods in Physics Research Section B. 71:228–233.
- Appleby PG, Nolan PJ, Gifford DW, Godfrey MJ, Oldfield F, Anderson NJ, Battarbee RW. 1986.  $^{210}\text{Pb}$  dating by low background gamma counting. Hydrobiologia. 141:21–27.
- Beike AK, von Stackelberg M, Schallenberg-Rüdinger M, Hanke ST, Follo M, Quandt D, McDaniel SF, Reski R, Tan BC, Rensing SA. 2014. Molecular evidence for convergent evolution and allopolyploid speciation within the *Physcomitrium-Physcomitrella* species complex. BMC Evolutionary Biology. 14:158. doi:10.1186/1471-2148-14-158.
- Bergamini A, Bisang I, Hodgetts N, Lockhart N, van Rooy J, Hallingbäck T. 2019. Recommendations for the use of critical terms when applying IUCN red-listing criteria to bryophytes. Lindbergia. 42:1–5.
- Blockeel TL, Bosanquet SDS, Hill MO, Preston, CD. 2014. Atlas of British and Irish bryophytes. Newbury: Pisces Publications.
- Bridge T. 2005. Controlling New Zealand pygmyweed *Crassula helmsii* using hot foam, herbicide and by burying at Old Moor RSPB Reserve, South Yorkshire, England. Conservation Evidence. 2:33–34.
- Callaghan DA. Submitted. IUCN Red List of bryophytes in Britain. Journal of Bryology.
- Callaghan DA, Medina R, Masson J, During H. 2020. Population status and ecology of the episodic moss *Physcomitrium eurystomum* Sendtn. in Britain. Journal of Bryology. 42:246–257.
- Denys L, Packet J, Jambon W, Scheers K. 2014. Dispersal of the non-native invasive species *Crassula helmsii* (Crassulaceae) may involve seeds and endozoochorous transport by birds. New Journal of Botany. 4:104–106.

- Ducker BFT, Warburg EF. 1961. *Physcomitrium eurystomum* Sendtn. in Britain. Transactions of the British Bryological Society. 4:95–97.
- Eckstein J. 2006. Die Moosdiasporenbanken einiger Teiche im ostthüringer Buntsandsteingebiet. Herzogia. 19:341–351.
- Funke T, Han H, Healy-Fried ML, Fischer M, Schönbrunn E. 2006. Molecular basis for the herbicide resistance of Roundup Ready crops. Proceedings of the National Academy of Sciences. 103:13010–13015.
- Furness SB, Hall RH. 1981. An explanation of the intermittent occurrence of *Physcomitrium sphaericum* (Hedw.) Brid. Journal of Bryology. 11:733–742.
- Gassmann A, Cock MJ, Shaw R, Evans HC. 2006. The potential for biological control of invasive alien aquatic weeds in Europe: a review. Hydrobiologia. 570:217–222.
- Herrera-Pantoja M, Hiscock KM, Boar RR. 2012. The potential impact of climate change on groundwater-fed wetlands in eastern England. Ecohydrology. 5:401–413.
- Hodgetts NG, Lockhart N, Lönnell N, Rothero G, Schröck C, Untereiner A, Vanderpoorten A. 2019. *Physcomitrium eurystomum*. The IUCN Red List of Threatened Species 2019: e.T85841752A87795278. Downloaded on 02 October 2019.
- Hussner A, Stiers I, Verhofstad MJJM, Bakker ES, Grutters BMC, Haury J, Van Valkenburg JLCH, Brundu G, Newman J, Clayton JS, Anderson LWJ. 2017. Management and control methods of invasive alien freshwater aquatic plants: a review. Aquatic Botany. 136:112–137.
- Kearse M, Moir R, Wilson A, Stones-Havas S, Cheung M, Sturrock S, Buxton S, Cooper A, Markowitz S, Duran C, Thierer T, Ashton B, Meintjes P, Drummond A. 2012. Geneious Basic: an integrated and extendable desktop software platform for the organization and analysis of sequence data. Bioinformatics. 28:1647–1649.
- Leach J, Dawson H. 2000. Is resistance futile? The battle against *Crassula helmsii*. Journal of Practical Ecology and Conservation. 4:7–17.
- Malkowsky Y, Ostendorf AK, Roth-Nebelsick A. 2018. Funariaceae underground – a spore bank for *Physcomitrella patens* and *Physcomitrium eurystomum*. Herzogia. 31:791–797.
- MBG. 2019. Tropicos, botanical information system at the Missouri Botanical Garden. Retrieved from: <http://www.tropicos.org>.

McDaniel SF, Von Stackelberg M, Richardt S, Quatrano RS, Reski R, Rensing SA. 2010. The speciation history of the *Physcomitrium*—*Physcomitrella* species complex. *Evolution*. 64:217–231.

Medina R, Johnson M, Liu Y, Wicket N, Shaw AJ, Goffinet B. 2019. Phylogenomic delineation of *Physcomitrium* (Bryophyta: Funariaceae) based on targeted sequencing of nuclear exons and their flanking regions rejects the retention of *Physcomitrella*, *Physcomitridium* and *Aphanorrhagma*. *Journal of Systematics and Evolution*. 57:404–417.

Müller J, Müller KF, Neinhuis C, Quandt D. 2006. PhyDE - Phylogenetic Data Editor. Available at: <http://www.phyde.de>.

Newmaster SG, Bell FW. 2002. The effects of silvicultural disturbances on cryptogam diversity in the boreal-mixedwood forest. *Canadian Journal of Forest Research*. 32:38–51.

Newmaster SG, Bell FW, Vitt DH. 1999. The effects of glyphosate and triclopyr on common bryophytes and lichens in northwestern Ontario. *Canadian Journal of Forest Research*. 29:1101–1111.

Nieuwkoop JAW. 2016. Standplaatsen van *Physcomitrium eurystomum* (eirond knikkertjesmos) langs de Waal. *Buxbaumiella*. 105:6–9.

Nováková E, Jiroušek M, Musil Z, Štěpánková P. 2015. Botanical survey and successional changes of vegetation in pools after restoration project in wetland near the Cisarska cave, Moravian Karst. In: MendelNet. Proceedings of International PhD Students Conference. Brno: Mendel University. pp 68–73.

Oesau A. 2008. Wuchsanomalien an Moosen in herbizidbehandelten Weinbergen Rheinhessens (Rheinland-Pfalz). *Archive for Bryology*. 28:1–7.

Pihakaski S, Pihakaski K. 1980. Effects of glyphonosate on ultrastructure and photosynthesis of *Pellia epiphylla*. *Annals of Botany*. 46:133–141.

Porley RD. 2013. England's rare mosses and liverworts. Princeton: Princeton University Press.

Poschlod P. 1993. "Underground floristics" – keimfähige diasporen im boden als beitrage zum floristischen Inventar einer landschaft am beispiel der teichbodenflora. *Natur und Landschaft*. 68:155–169.



Rimac A, Šegota V, Alegro A, Koletić N, Vuković N, Papp B. 2019. New and noteworthy bryophyte records from lacustrine drawdown zones in Croatia. *Herzogia* 32:315–325.

Rodwell JS. (ed.). 2000. *British plant communities: aquatic communities, swamps and tall-herb fens*. Cambridge: Cambridge University Press.

Sims PF, Sims LJ. 2016. Control and eradication of Australian swamp stonecrop *Crassula helmsii* using herbicide and burial at two ponds at Mile Cross Marsh, Norfolk, England. *Conservation Evidence*. 13:39–41.

Stjernquist I. 1981. Photosynthesis, growth and competitive ability of some coniferous forest mosses and the influence of herbicides and heavy metals (Cu, Zn). Unpublished thesis, University of Lund, Lund, Sweden.

Swann EL. 1982. Norfolk bryophytes today. *Journal of Bryology*. 12:77–112.

Touw A, Rubers WV. 1989. *De Nederlandse bladmossen*. Utrecht: KNNV.

van der Loop JM, Hoop LD, van Kleef HH, Leuven RS. 2018. Effectiveness of eradication measures for the invasive Australian swamp stonecrop *Crassula helmsii*. *Management of Biological Invasions*. 9:343–355.

van Kleef HH, Brouwer E, van der Loop JMM, Buiks M, Lucassen ECHET. 2017. *Systeemgerichte bestrijding van watercrassula*. Nijmegen: Stichting Bargerveen.

Table 1.  $^{210}\text{Pb}$  concentrations and chronology in the sediment core taken in October 2019 from Langmere, Norfolk. Values are shown to three significant figures.

Sample	Depth (cm)	Dry mass (g cm <sup>-2</sup> )	Total $^{210}\text{Pb}$ (Bq Kg <sup>-1</sup> ) (SE)	Supported $^{210}\text{Pb}$ (Bq Kg <sup>-1</sup> ) (SE)	Unsupported $^{210}\text{Pb}$ (Bq Kg <sup>-1</sup> ) (SE)	Cumulative unsupported $^{210}\text{Pb}$ (Bq Kg <sup>-1</sup> ) (SE)	Chronology		Sedimentation rate		
							Date (AD)	Age (yr) (SE)	g cm <sup>-2</sup> yr <sup>-1</sup>	cm yr <sup>-1</sup>	± %
A01	0–1	0.489	177 (15.6)	23.4 (3.85)	154 (16.1)	880 (71.2)	2013	6 (3)	0.090	0.093	10.3
A02	1–2	1.46	193 (19.6)	28.4 (4.76)	165 (20.1)	2430 (182)	1999	20 (3)	0.0548	0.054	11.8
A03	2–3	2.50	116 (15.2)	28.1 (3.97)	87.4 (15.7)	3700 (275)	1981	38 (4)	0.0581	0.051	23.5
A04	3–4	3.73	57.5 (5.92)	36.5 (1.82)	20.9 (6.19)	4270 (321)	1967	52 (6)	0.158	0.125	36.2
A05	4–5	5.04	40.9 (6.46)	31.0 (1.98)	9.88 (6.76)	4460 (331)	1961	58 (7)	0.274	0.21	72.1
A06	5–6	6.34	38.4 (4.76)	36.9 (1.53)	1.46 (5.00)	4520 (341)	1957	62 (7)	0.356	0.276	93.3
A07	6–7	7.62	51.8 (6.42)	36.5 (1.98)	15.3 (6.72)	4600 (349)	1955	64 (8)	0.150	0.116	49.7
A08	7–8	8.92	51.6 (5.85)	37.8 (1.82)	13.8 (6.13)	4790 (359)	1946	73 (9)	0.124	0.095	52.7
A09	8–9	10.2	40.2 (5.04)	34.1 (1.54)	6.18 (5.27)	4910 (367)	1938	81 (11)	0.213	0.165	91.1
A10	9–10	11.5	46.0 (6.23)	31.1 (1.55)	14.9 (6.42)	5040 (374)	1926	93 (13)	0.0621	0.048	58.3
A11	10–11	12.8	39.6 (4.75)	27.4 (1.44)	12.2 (4.96)	5210 (383)	1898	121 (26)	0.0316	0.025	64.6
A12	11–12	14.0	46.8 (5.41)	31.2 (1.46)	15.6 (5.60)	5380 (388)					
A13	12–13	15.3	18.9 (9.37)	29.3 (1.41)	-10.4 (9.48)						

Table 2. Artificial fallout radionuclide concentrations in the sediment core taken in October 2019 from Langmere, Norfolk. Values are shown to three significant figures.

<b>Sample</b>	<b>Depth (cm)</b>	<b><sup>137</sup>Cs (Bq Kg<sup>-1</sup>) (SE)</b>	<b><sup>241</sup>Am (Bq Kg<sup>-1</sup>) (SE)</b>
A01	0–1	45.0 (3.56)	0
A02	1–2	50.3 (4.37)	3.24 (1.64)
A03	2–3	50.8 (3.86)	0
A04	3–4	24.8 (1.39)	0
A05	4–5	22.6 (1.54)	0
A06	5–6	24.8 (1.15)	0
A07	6–7	25.2 (1.59)	0
A08	7–8	21.3 (1.32)	1.24 (0.61)
A09	8–9	18.0 (1.05)	0
A10	9–10	15.9 (1.01)	0
A11	10–11	12.9 (0.93)	0
A12	11–12	12.4 (0.90)	0
A13	12–13	9.45 (0.83)	0

## Figure captions

Figure 1. Fallout radionuclide concentrations in sediment core taken from Langmere, Norfolk, showing (a) total  $^{210}\text{Pb}$ , (b) unsupported  $^{210}\text{Pb}$ , and (c)  $^{137}\text{Cs}$  and  $^{241}\text{Am}$  concentrations versus depth.

Figure 2. Radiometric chronology of sediment core taken from Langmere, Norfolk, showing the CRS model  $^{210}\text{Pb}$  dates and sedimentation rates. The solid line indicates ages and the dashed line shows sedimentation rates.

Figure 3. Shoots and sporophytes of *Physcomitrium eurystomum* arising from the germination trial of one of the soil samples.

Figure 4. Germination of shoots of *Physcomitrium eurystomum* from different sediment layers of three soil cores taken from Langmere, Norfolk. Abundance is categorised as (1) <10 shoots, (2) 10–100 shoots and (3) >100 shoots.



