

SHORT REPORT

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Microcomputer tomography (microCT) as a tool in *Pinus* tree breeding: pilot studies

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Abstract

Background: *Pinus* breeding programmes yield high numbers of seeds, but a non-destructive method to determine seed viability is still lacking. With the long reproductive cycle (up to 28 months) of *Pinus* species, determining when fertilisation occurs can assist when applying tissue culture methods like somatic embryogenesis (SE). For SE, pre-cotyledonary zygotic embryos are ideal for culture initiation (some weeks after fertilisation), while mature zygotic embryos are extracted and used for in vitro amplification during organogenesis. Achieving automated viability assays in extracted seeds would also be helpful, as would being able to find numbers and condition of seeds in immature or unopened cones. For such applications, microcomputer tomography (microCT) was a candidate technology.

MicroCT was used to scan immature cones of *Pinus radiata* D.Don at four-weekly intervals after pollination to determine time of fertilisation. After harvesting of mature cones, a sample of 30 seeds of each of three *Pinus* species (*P. radiata*, *P. patula* Schiede ex Schltdl. & Cham. and *P. pinaster* Aiton) were scanned together in one microCT scan, and the same seed was allowed to germinate on moist filter paper after scanning. In addition, a mature unopened cone of *P. radiata* was also microCT-scanned to determine the potential for determining seed viability in situ, and the seed was subsequently extracted for examination.

Findings: Fertilisation evidently occurred between weeks 64 and 68 after pollination in *P. radiata*. The microCT data for extracted seeds of the three species identified some seeds with large voids, which suggest non-viability (low to zero germination potential), and that was confirmed by the germination test. In addition to demonstrating in situ seed viability in the unopened *P. radiata* cone, the seed extraction revealed that some non-viable seeds remained trapped in the basal part of the dried cone.

Conclusions: This case study demonstrates the feasibility of using microCT scans as a partially non-destructive method in *Pinus* tree breeding programmes to confirm fertilisation and determine seed viability. However, scanning the seed after extraction from mature cones can help to determine viability which is often hard to gauge from seeds' external appearance. Future studies need to narrow the time window of fertilisation by scanning a wider variety of genotypes between weeks 64 and 68 after pollination.

Keywords: X-ray tomography, Tree breeding, Non-destructive analysis, microCT, Seed set, *Pinus radiata*

Background

The development of female reproductive structures of *Pinus* species can be divided into three general phases: first seasonal growth during which pollination occurs, second seasonal growth during which fertilisation occurs and a period of cone maturation (Sweet and Bollmann 1971; Williams 2009; Fernando 2014). The female

strobilus contains ovules that become seeds after being fertilised by pollen during the second seasonal growth phase. Seed development and cone maturation are rapid after fertilisation (Bramlett et al. 1977; Williams 2009). During seed and cone maturation, density differences between different tissues are more evident, for example, the formation of the hard seedcoat (Bramlett et al. 1977). Microcomputed tomography (microCT) scans can be used as a non-destructive method to determine differences based on these density differences between tissues, as indicated in previous studies (du Plessis et al. 2016;

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Guelpa et al. 2016). The number of seed and viability depends on the type of crosses performed. Interspecific crosses, for example, yield far fewer viable seeds than do intraspecific crosses (Williams 2009).

Although pollination and fertilisation in *Pinus* species may yield a large number of mature cones filled with seed, some might be non-viable due to pollination failure or post-zygotic degeneration (Owens et al. 1990; El-Kassaby et al. 1993). Seeds need to be extracted, and viable seeds separated from non-viable ones (Bramlett et al. 1977) before sowing. Generally, this is done using one of the following methods: a water-soaking test; shaking in a cone tumbler (Fry and Stephens 2013); use of an optical sorting machine (Delwiche et al. 2005) and pressure vacuum (Lestander and Bergsten 1985); X-radiography (International Seed Testing Association 1999) or the Incubation Drying & Separation technique (IDS) (Simak 1981, 1984; Bergsten 1988; Bergsten and Sundberg 1990; Downie and Bergsten 1990; Downie and Wang 1992; Falleri and Pacella 1997; Demelash et al. 2002). Viability of seeds is determined according to parameters such as colour, weight and size, often using lasers and/or other sensors as opposed to strictly manual sorting, but success may vary among species (Tigabu and Odén 2003).

The number of mature seed is used as an indicator for interspecific *Pinus* hybridisation success although a high number of these seeds could be non-viable (Critchfield and Kinloch 1986; Critchfield 1975). There is a need to identify a reliable, repeatable and non-destructive method that could quantify successful seed set (viable seed) and to confirm how many weeks after pollination fertilisation takes place. This can provide valuable information for tissue-culture programmes (such as somatic embryogenesis or organogenesis), which are applied in agriculture with increasing success (Balla and Mansvelt 2013; Mansvelt et al. 2013), and might improve interspecific hybridisation success with *Pinus* species (Reed 2004; Mehetre and Aher 2004; Lelu-Walter and Pâques 2009; Montalbán et al. 2010; Montalbán et al. 2011).

The reproductive cycle of *Pinus radiata* is up to 28 months long, and fertilisation occurs around 16 months after pollination (Sweet and Bollmann 1971). After fertilisation, seeds are already full size but will still develop until the cones are harvested at around 28 months (Bramlett et al. 1977). Previous studies indicated that mature seed is visible in X-ray radiograph pictures (Bramlett et al. 1977). These images are two-dimensional (2D) and can be used for diagnosis but are limited in contrast and cannot be used for quantitative analysis (Tigabu and Odén 2003). In contrast, microCT scans provide a three-dimensional (3D) model, which generates more accurate and reliable data.

Computer tomography (CT) and microCT scanning methods were originally developed for use by the medical profession as a diagnostic tool to obtain a 3D image in a non-destructive manner (Stuppy et al. 2003; Staedler et al. 2013). It is not yet widely applied in plant sciences (Kalathingal et al. 2007; van der Niet et al. 2010) even though it is a relatively simple non-destructive technique for gaining insight into smaller objects or plant tissues and for helping to understand phenotypic information (total sum of the observable characteristics of an organism) (Stuppy et al. 2003; Staedler et al. 2013; Dhondt et al. 2010). During microCT scanning, thousands of 2D X-ray images are combined to construct an accurate 3D model. The data, based on differential X-ray attenuation, are analogous to those otherwise obtainable only by serial sectioning of plant tissues (Stuppy et al. 2003; van der Niet et al. 2010; Staedler et al. 2013). It requires no staining, sectioning or fixing but produces a 3D digital map (viewed from an arbitrary angle) of the specimen that allows measurements and visualisations (Stuppy et al. 2003; van der Niet et al. 2010; Staedler et al. 2013), for example, of metabolite content, pollination status or crop yield (Staedler et al. 2013; van der Niet et al. 2010). Previous experiments have been able to distinguish between pith, xylem, cortex, vascular bundles, leaf bases, seeds and ovuliferous scales (Pika-Biolzi et al. 2000). This is due to contrasting plant tissues (soft versus hard) absorbing X-rays differently due to different thicknesses of cell walls and cell contents (Stuppy et al. 2003). Previous studies have classified *Pinus patula* seeds as viable or not using near-infrared transmittance (energy in the region of the electromagnetic radiation spectrum at wavelengths longer than those of visible light but shorter than those of radio waves) and reflectance spectroscopy (study of light as a function of wavelength) (Tigabu and Odén 2003). However, qualitative and quantitative investigation of the internal morphology and histology of plants is a potential application of microCT scans (Stuppy et al. 2003).

MicroCT has progressed to the point where service facilities are available worldwide and especially in university departments, making the method easily accessible and more cost-effective. Its use in non-destructive testing of various types of materials has made it a valuable tool in materials sciences (Maire and Withers 2014), geosciences (Cnudde and Boone 2013) and agricultural and food sciences (Schoeman et al. 2015). The method is very diverse and can provide quantitative information on density of biological materials such as maize kernels (Guelpa et al. 2015) to determine, for example, milling quality. It has also proved possible to scan large numbers of samples in a single operation, making high-throughput analysis feasible for this type of application (Guelpa et al. 2016).

This study investigated microCT scans as an alternative non-destructive method in *Pinus* breeding programmes. Three experiments were conducted to determine whether microCT scans can be used to confirm at what time (weeks after pollination) fertilisation occurred, whether seed viability can be assessed by scanning multiple seeds together and whether viable seed can be counted in a closed *P. radiata* cone.

Methods

The following general considerations in the three experiments were:

- Different stages of the *Pinus* female flower are defined as female strobilus (up to pollination), conelet (between pollination and fertilisation) and cone (after fertilisation till harvesting) (Williams 2009).
- Seeds were numbered with a permanent marker to compare results between experiments.

Experiment 1: time of fertilisation

Controlled crosses were performed in a *P. radiata* seed orchard at Karatara (close to Knysna, South Africa) during the winter months (July and August 2013). Female strobili were pollinated with *P. radiata* pollen lots following the standard pine pollination protocol (Bramlett and O'Gwynn 1981; Williams 2009; Huyn 1976; Slee and Abbott 1990; Critchfield and Kinloch 1986). Three conelets were harvested at weekly intervals for the first 8 weeks after pollination and thereafter every fourth week until week 104 after pollination. Week 104 represents 22 months after pollination when cones are considered mature (Bramlett and O'Gwynn 1981). A total of 100 conelets were harvested throughout this study, fixed in an aqueous solution of formalin (10% v/v), acetic acid (5% v/v) and ethanol 50% v/v of 96% alcohol) (Mert and Soylu 2006; Coetzee 1982; Sass 1958) in sealed glass bottles (Staedler et al. 2013) and couriered to Stellenbosch within 24 h of harvesting. These glass bottles were stored at room temperature (24 °C) and out of direct sunlight until microCT scans commenced. Additional data collected were fresh weight, length and width of conelets (Sweet and Bollmann 1971).

Conelets were subjected to X-ray microcomputed tomography scans using a Phoenix V|Tome|X L240/NF180 scanner (General Electric, USA). X-ray settings for a single scan were 100 kV and 200 μ A, with 1700 images being acquired in a full rotation at image acquisition time of 500 ms per image, with no averaging and no skipping of images (Broeckhoven et al. 2016; du Plessis et al. 2016). Dose rate was measured in kilovolts based on a previous study conducted by Broeckhoven et al. (2016). Reconstruction was undertaken with system-

supplied Datos reconstruction software. Analysis was performed with VGStudio Max 2.1 and 2.2 software (Volume Graphics, Heidelberg, Germany).

Experiment 2: number of viable seed by scanning groups of seeds in three species

Seed from *P. radiata*, *P. patula* and *P. pinaster* were obtained from commercial nurseries. Thirty seeds from each species were subjected to microCT scans (total of 90 seeds) to determine percentage porosity. Seeds were subjected to microCT scans (Phoenix V|Tome|X L240/NF180) with 60 kV and 240 μ A for the seed scans of 30 seeds in one scan, at a voxel resolution of 24 μ m. After microCT scans, the seeds were subjected to a water-floating test to verify viability. Seeds were placed in overnight glass beakers filled with distilled water at room temperature (25 °C). The number of submerged seeds were counted and taken as viable. Seeds were then placed between moistened filter papers at room temperature and natural light (16:8 h light/dark). Seeds were taken as germinated when the radicle was at least 10 mm. Germination percentages were documented for 4 weeks.

Experiment 3: number of viable seed by scanning an intact *Pinus radiata* cone

One arbitrarily chosen mature *P. radiata* cone (closed) with seeds was harvested from the top of a tree and scanned to investigate the potential for in situ seed viability assessment. The pine cone scan (Phoenix V|Tome|X L240/NF180) was done at 120 kV and 150 μ A at a voxel resolution of 80 μ m. Image averaging and skipping was used to enhance image quality. Detector shift was activated to minimise rotation artefacts, and automatic scan optimisation was activated. Reconstruction was done with system-supplied software. The scan times were 1 h each including setup time.

Image analyses for experiments 1 and 2 were done with VGStudioMax 2.2 software (volume graphics). The basic processing used involved an adaptive Gauss filter to remove noise and to select the material of interest using region growing and morphological operations. This process involves the processing of CT slice images by selecting the background air with a 'flood fill' tool in 3D. After viewing a number of slice images to ensure the selection was done properly, the air was removed. Similarly, each seed is then selected to include its internal air volumes (Matthews and du Plessis 2016). None of this is required for a basic viewing of slice images in a typical non-destructive analysis viewing but for 3D analysis and quantitative volume measurements as reported here. After scanning, seed germination was determined as described in Experiment 2.

Results

Experiment 1: time of fertilisation

Conelets scanned at weeks 0 (24 h after pollination), 7, 60 and 64 (14 to 15 months after pollination) had a uniform grey colour with no distinct visible density differences. Since X-ray absorption, which is used in microCT, depends on physical density, it is possible to infer density differences based on grey value differences.

Approximately 15 to 16 months after pollination (week 68), full-sized seeds, the central axis and ovuliferous scales were clearly visible, indicating that fertilisation occurred between weeks 64 and 68 (Fig. 1). Various stages of seed development and an increase in the number of viable seeds were observed in conelets scanned between weeks 68 and 104 (mature cones). A steady increase in fresh weight (g), length (mm) and width (mm) of the conelets between weeks 60 and 104 indicated normal growth of conelets (Table 1). The mean fresh weight at weeks 60 and 104 were 15.6 and 208.1 g, respectively. The average length along the geometric axis of conelets increased from 36 mm (week 60) to 118.2 mm (week 104), while the average width increased from 26.3 mm (week 60) to 64.2 mm (week 104).

Experiment 2: number of viable seed by scanning a cluster of seed

During the water-soaking test, 30 seeds of *P. radiata*, 29 of *P. patula* and 28 of *P. pinaster* submerged, indicating a seed viability of 100, 97 and 93%, respectively. Germination percentage of seeds kept in moistened filter papers (up to 4 weeks) matched the results of the water-soaking test. The data collected with the microCT scans also mirrored the results from the water-soaking test and germination filter-paper test (seeds were numbered). Dark seeds had lower weights and floated on water, indicating a large air volume (Figs. 2 and 3), and these seeds proved to be non-viable. Using colour coding to represent air volume also indicated that non-viable seed had an air volume higher than 20 mm³ (shown in pink in

Table 1 Fresh weight and size of *P. radiata* cones harvested between weeks 60 and 104

Week (n = 3)	Fresh weight (g)	Length (mm)	Width (mm)
60	15.6	36.0	26.3
64	22.7	45.3	29.7
68	72.1	72.7	44.3
72	101.6	86.0	40.8
76	149.9	93.0	44.5
80	173.4	97.3	47.3
104	208.1	118.2	64.2

Fig. 3). The percentage porosity (air inside seed) of the seeds that did not submerge was more than 50%. Viable seeds of *P. radiata* had an average porosity of 17.3%, *P. patula* 22.7% and *P. pinaster* 17.5%.

Experiment 3: number of viable seed by scanning an intact *Pinus radiata* cone

Images from the *P. radiata* cone scanned with intact seed indicated non-viable seeds which were easily distinguished from viable seeds (Fig. 4). Unfortunately, the seeds were not able to be numbered in situ, so it was difficult to distinguish between viable and non-viable seeds after shaking from the cone. Care should be taken to extract all the seed as some non-viable seed seemed to get trapped inside the cone (bottom of cone with unopened scales). This might be due to the scales that did not open sufficiently enough for the seeds to be released. However, the number of viable seeds corresponded with the water-soaking test.

Discussion

Mature seed cones from interspecific hybrid crosses are the first 'product' in the sequential process of tree breeding as it contains the *F*₁ progeny (seed). The number of seed harvested is generally considered an indication of breeding success (Johnsson 1976). However, pre-zygotic

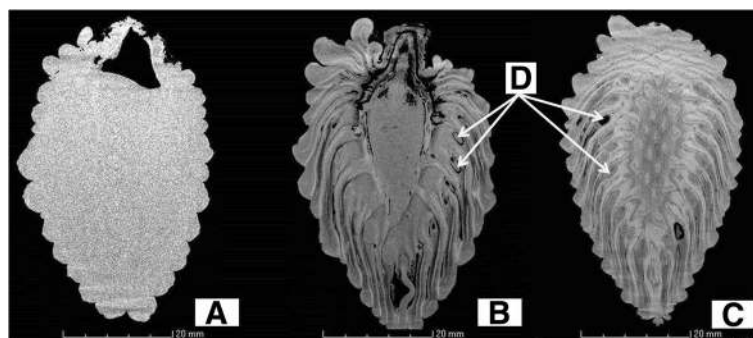


Fig. 1 Conelets microCT scanned (3D longitudinal cuts) at week 64 (a) and cones at weeks 68 (b) and 80 (c) indicating fertilisation occurred after week 64 with full-sized seeds visible (d) at weeks 68 and 80

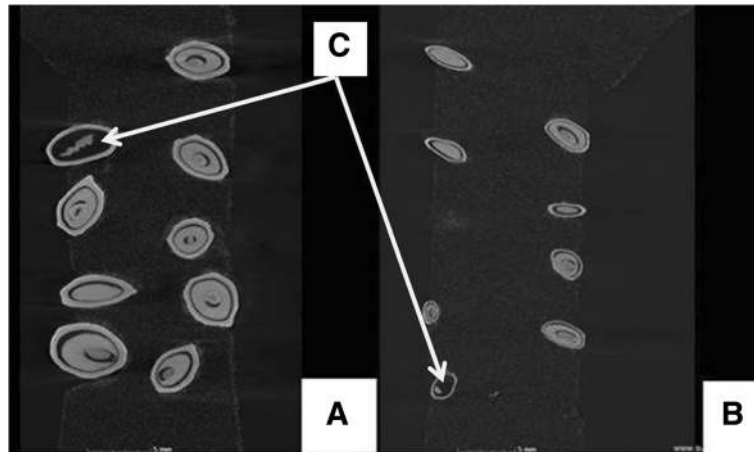


Fig. 2 A microCT slice image showing *Pinus pinaster* (a) and *P. patula* (b) seeds, with one seed in each case having a large void (black space) indicating it is non-viable (c)

barriers (before fertilisation) can reduce the numbers of mature cones or seed harvested, while post-zygotic barriers (after fertilisation) can increase the number of hollow seeds without affecting the number of seed extracted from the cones (Critchfield and Kinloch 1986; Owens et al. 1990). Therefore, estimating the time (in weeks) after pollination that fertilisation might occur can help to estimate viable seed set during interspecific hybridisation and make subsequent tissue-culture (embryo rescue and micropropagation) projects more effective (Cisneros and Tel-Zur 2010). Previous studies used radiography to determine whether mature seed can be viable (Bramlett et al. 1977; Tigabu and Odén 2003). However, they could only determine whether seeds are

full, partially full or empty and could not predict with certainty whether these seed might be viable. Full seeds were thus classified as potentially sound seed but damaged full seeds (insects, aborted ovules, etc.) could not be distinguished from healthy full seeds.

This study investigated microCT scans as a prospective tool in tree breeding using three different types of experiments to refine the technique of estimating fullness and viability of seeds. The advantages of microCT scans are the non-destructive imaging capability and quantitative analysis capabilities, for example measuring porosity in seeds. Although it is a non-destructive imaging method, the sampling process is destructive in that conelets are 'destroyed' because they cannot be grafted back onto the

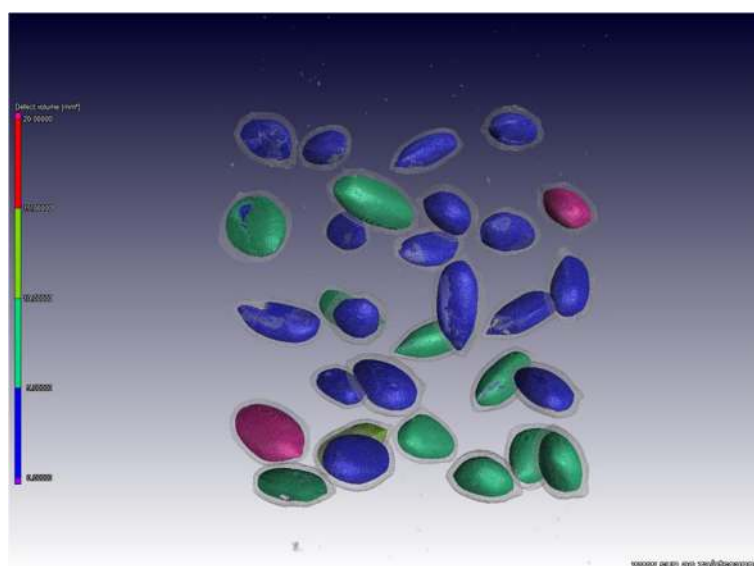


Fig. 3 A 3D image of 30 *Pinus pinaster* seeds with the voids colour coded, indicating two non-viable seeds with voids larger than 20 mm³ (pink). The colour coding is based on volume; therefore, blue and green seeds have intermediate volumes according to the colour bar

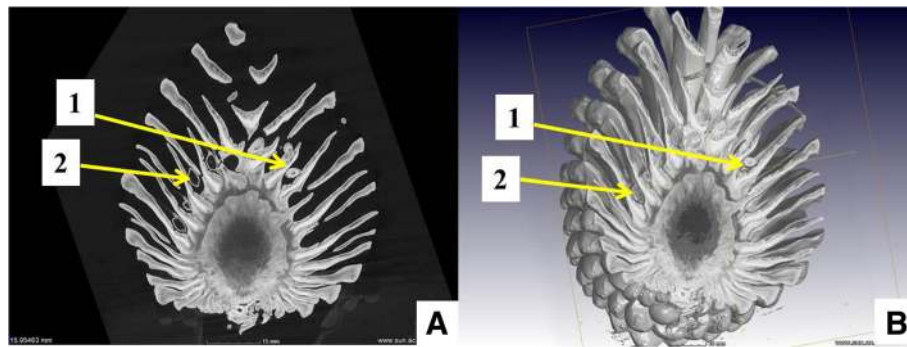


Fig. 4 Demonstration of intact pine cone (week 104) microCT scan in slice (a) and 3D virtual cut-open (b) images, with non-viable seeds *upper left* (1) and viable seeds *top right* (2) as indicated by their voids

trees to develop into mature seed cones. However, reconstructed images create a permanent, tangible record and life-like image for future analysis, comparison or prediction studies (Perrin et al. 2010; van der Niet et al. 2010; Staedler et al. 2013). Seed damage due to insects or aborted ovules will be clearly visible. Furthermore, X-ray absorption during CT scans depends on physical density and based on grey value differences (du Plessis et al. 2013; Lindgren et al. 2016).

Fertilisation in *Pinus* pure species and hybrids are estimated to take place between 10 (Fernando et al. 2005) to 16 months after pollination (Sweet and Bollmann 1971). The results from Experiment 1 indicated that fertilisation occurred between weeks 64 and 68 (15 to 16 months after pollination) in *P. radiata*, narrowing down the known time of fertilisation. Therefore, seed set and seed damage could be estimated as early as week 68 (15 to 16 months after pollination). To confirm precisely when fertilisation happens and perform reliable seed set counts in *P. radiata*, different clones will need to be microCT scanned between weeks 63 and 69 after pollination to narrow down the time of fertilisation.

Experiments 2 and 3 investigated whether microCT scans can be used as an alternative method to determine seed set and viability accurately and measurably (Kalathingal et al. 2007). Studies with microCT scans are very limited in plant sciences, due to plant tissues mostly consisting of light elements which display low X-ray absorptions. However, this can be compensated with very long scanning times but with decreasing quality due to the probability of motion artefacts. The results of the seed viability tests indicated clear differences between seed (viable versus non-viable) by quantitative porosity measurements indicated by the colour-coded 3D images. When mature seed cones are harvested in large quantities, this can be a useful tool to distinguish between cones with a high number of non-viable seed, lowering the cost of seed extraction.

Previous studies indicated that X-rays are absorbed to varying degrees by different parts of the seed and helps to distinguish between different tissues and injuries, although physiological changes are not visible (Simak 1957; Kriebel 1972). Very mild X-ray doses (~100 kV) do not injure physiologically sound (freshly collected) seed, and the germination ability should remain high (Simak 1957; Gustafsson and Simak 1957; Ohba and Simak 1961) but might differ between species (Gustafsson and Simak 1958). As mature seed is also less affected by mild X-ray doses (Gustafsson and Simak 1958; Johnsson 1976), this study was carried out only on mature seed with an X-ray dose of ~100 kV. Seed germination with moistened filter paper confirmed that mature seed scanned at ~100 kV X-ray doses were not damaged as germination was consistent with seed viability determined during experiment 2 (seeds were numbered). Seedling survival was, however, not determined the past 4 weeks after sowing. Broeckhoven et al. (2016) concluded that small doses of X-rays have no harming effect on live reptiles of various sizes. Previous studies also indicated that samples of up to 150 maize kernels yielded good survival results and allowed for the scanning of more than 1000 maize kernels per day with semi-automated analysis (du Plessis et al. 2016; Guelpa et al. 2016).

Conclusions

MicroCT was applied in a *Pinus* tree breeding study with three experiments. In the first experiment, the fertilisation time of *P. radiata* was narrowed down by microCT imaging to probably occur between weeks 64 and 68 after pollination. Full-sized seeds were visible at week 68, indicating initial seed set although not fully mature seeds. In the second experiment, quantitative porosity analyses of seeds were used to identify non-viable seed. This method can be used for qualitative viewing for faster and lower-cost application or more time-consuming quantitative analysis. The clarity of seed

viability assessment by microCT scans should allow its use for breeding assessments or calibration of indirect measures. An experiment with a full cone with viable and non-viable seed demonstrates the potential for in situ seed viability assessment. However, care should be taken to shake out all the seeds as some can still be trapped between scales at the bottom of the cones.

Abbreviations

2D: Two-dimensional; 3D: Three-dimensional; CT: Computed tomography; IDS: Incubation Drying & Separation technique; microCT: Microcomputer tomography

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Authors' contributions

The authors made the following contributions to the manuscript: (1) HH contributed to the experimental design, performed the crosses, harvested the cones, extracted the seed via cone drying, participated in the seed germination studies, carried out the collection and analysis of data and prepared and edited the manuscript relating to these sections. (2) AdP contributed to the sample mounting, scan setup optimization for best quality, image processing method development for best quantitative analysis and in the "Methods" part of manuscript. (3) SGIR contributed to the scanning and basic processing of 3D data as described and developed by AdP and assisted with the image processing method development at places. The final manuscript was reviewed and edited by all three authors. All authors read and approved the final manuscript.

Competing interests

The authors declare that they have no competing interests.

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