Quantiﬁcation of differences in germination behaviour of pelleted and coated sugar beet seeds using x-ray computed tomography (x-ray CT)

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Abstract
Seed enhancement technologies i.e. priming, pelleting and coating have been extensively used throughout the last century to improve crop yield and to reduce losses associated with pest infestation. However, until recently, it has not been possible to non-destructively assess the effect of seed enhancement technologies belowground due to the opacity of soil. Using x-ray computed tomography (x-ray CT) we undertook a 4D visualisation of the germination process of four different sugar beet seed enhancement treatments (untreated/naked, coated, pelleted and pelleted+coated) in soil. The aim of this study was to improve the understanding of the germination process in the natural environment of the seed to inform future soil management and seed enhancement processes. Using x-ray CT we were able to quantify the germination and establishment process of different seed enhancement technologies in soil non-destructively for the ﬁrst time. We observed a delay in seedling growth posed by the addition of a physical barrier, i.e. the seed coating. However, an enhanced radicle growth rate was observed in pelleted, as well as pelleted and coated seeds, after overcoming the physical barrier. The disadvantage posed by the addition of seed coating was overcome after four days of seedling growth. Further work should focus on reﬁnements to the type and composition of the pelleting which we observed to have a retarded effect on seed germination.

Introduction
With an increasing global population (ca 9.2 billion in 2050) and demand for food, there is a need to increase crop yield and efﬁciency across a wide range of plants (Lal 2013); however, deterioration of suitable agricultural land for crop production is a signiﬁcant problem (Monneveux et al 2013). Therefore, it is particularly important to identify crops that have a reduced need for nutrients and enhanced ability to overcome stress (Chapuis et al 2012) and to maximise yield. Crop seeds require certain soil properties (especially moisture and temperature) to initiate germination, a crucial stage which inﬂuences the success of establishment into full grown plants. The seeding material consists of a seed containing the perisperm and the embryo, as well as the surrounding fruitwall material. Both the seed and the fruitwall are referred to as the fruit. From this point the term ‘seed’ is not used in a botanically strict sense and includes both the fruitwall and the seed. Water absorption (imbibition) is the ﬁrst and fundamental step in germination process (William et al 1995). During imbibition, the dry seed hydrates and swells which increases seed volume. Additionally, the fruit wall starts to soften which allows the radicle to penetrate the fruit wall and emerge into the soil (Lundgren 2009). To enable the water absorption process from the soil, close seed–soil contact is important (Brown et al 1996). Germination occurs when embryo growth overcomes the constraints of the fruit wall surrounding the seed (Bewley et al 2013, Smýkal et al 2014). Seed technologies aim to sustain...
and improve the health and yield of crops as well as emergence and germination (Taylor et al 1998, Ehsanfar and Modarres-Sanavy 2003). Originally, seed enhancement technologies, i.e. seed pelleting, were used to ensure successful precision sowing or to synchronise male and female inbred seeds (Vyn and Murua 2001, Gorim 2014). In addition to this, different enhancement technologies aim to improve performance to overcome growth restricting influences of the soil caused by temperature or moisture extremes. This may be through seed coatings that supply nutrients (Silcock and Smith 1982, Masauskas et al 2008), hormones (Powell and Matthews 1988), peroxides for oxygen provision or polymer coatings with hydrophilic abilities (Vyn and Murua 2001, Gorim 2014). Seed coating processes can be divided into two groups: seed pelleting and film coating. Pelleting consists of the addition of a relatively thick layer of material (often wood fibre) which is typically intended to increase the total volume and roundness of the seed to enhance the accuracy of planting. The added material may also contribute to increased water uptake and maintain sufficient moisture content during germination. Film coatings do not change the shape of the seed and are used to apply pesticides and fungicides (Hill 1999).

Besides crop enhancement, our understanding of seed germination and seed–soil interaction is limited. Field trials provide information concerning how the plumule grows after germination (Chang et al 2004). These field trials are limited to aboveground observations and hence do not provide insight into the germination process as the initial step of plant establishment. A Rhizotron enables a single slice 2D view of a root system facilitated by a glass window (Klepper and Kaspar 1994). The resulting root structure however, is influenced by the boundaries posed by the glass windows which influences the growth behaviour and eventually the root architecture. Observation of seed germination is impeded as it is unlikely that radicle growth is observed through the glass window. If the seed would be placed next to the glass layer, the seed opening and therefore the germination process, would be influenced. X-ray computed tomography (x-ray CT), a non-invasive and non-destructive 3D imaging technique, has been increasingly used to visualise soil properties like porosity (Vogel et al 2010) and in situ root systems (Tracy et al 2013, Mairhofer et al 2013). Magnetic resonance imaging and x-ray CT have previously been used for 3D root quantifications and it has been reported that x-ray CT is better suited for smaller pot diameters (Metzner et al 2015). However, only few studies have been undertaken to examine seed germination in soil especially regarding the impact of seed coatings. Most previous work on seed germination have been conducted on seeds grown without soil (Gagliardi and Marcos-Filho 2011, Galhaut et al 2014, Devarrewaere et al 2015). These studies have shown that it is possible to visualise structural changes within the seed during germination. Gregory et al (2003) was the first to use x-ray CT to image wheat seedling growth in soil with a resolution of 100 μm verifying the suitability of this technology to monitor seedling establishment in situ. Soil generally has an opaque and heterogeneous structure which has limited our ability to observe germination in situ (Brown et al 1996). Galhaut et al (2014) previously showed tissue detachment between radicle and cotyledons contributing to hydration which was not previously observed in unprimed seeds using x-ray CT (however, crucially not in soil).

Here we report the application of x-ray CT to quantify the impact of four seed enhancement technologies (naked (N), film coated (FC), pelleted (P) and pelleted and film coated (P+FC)) on the spatio-temporal germination of sugar beet seeds grown in soil. The aim of this study was to assess differences in performance of different seed enhancement technologies to illustrate the viability of x-ray CT for future germination studies in soil. Treatments were chosen based on commercial interest (pelletted+coated) divided into their basic components (coating and pelleting) compared to the untreated seed (naked). It was hypothesised that the enhanced seeds with an applied pelleting would have a germination advantage over the naked seeds whereas coated seeds might show a disadvantage due to the effect of the pesticide components.

**Materials and methods**

A Dunnington Heath series sandy loam soil (66.4% sand, 18.0% silt, 15.6% clay and 4.53% organic matter) was collected from The University of Nottingham farm at Bunny, Nottinghamshire (52.8586°, −1.1280°), air-dried and sieved to <1 mm. Sugar beet seeds were supplied by Syngenta Seeds AB, Sweden. Four different treatment types have been chosen based on commercial availability using naked (uncoated) (N), film coated (FC), pelleted (P) as well as pelleted and film coated seeds (P+FC). The pelleted treatment is a Syngenta in-house development mainly consisting of woodmeal and clay. Seeds are coated with a standard fungicide and insecticide treatment. The precise coating and pelleting compositions are treated confidentially. All used seed treatments can be ordered at Syngenta Seeds AB, Sweden, by referring to this study.

It was imperative that different column packing methods had to be first pre-tested to ensure maximum comparability of field conditions as well as accurate reproducibility. Method A displayed in figure 1 facilitates wetting via capillary action. An initial amount of dry soil was poured into the column and saturated with water from the bottom. The seed was placed centrally placed on the bottom layer and covered with dry soil. Capillary action saturated the top layer after a few minutes. In method B, the entire column was filled with dry soil and saturated with
water. Then, a hole of the size of the seed was created, the seed placed into the hole and filled with dry soil. Method C was similar to method A but conducted only with dry soil. Therefore, the column was filled with dry soil, saturated and the seed placed centrally on the layer. A second layer is poured onto the seed and the capillarity force saturates the second layer. (B) The column is filled completely with dry soil and saturated. A hole in the size of the seed is dug and the seed placed into the hole. The hole is filled with dry soil and capillarity force saturates the filling. (C) A first layer is packed dry, the seed sown on top the layer and a second layer applied dry as well. The whole column is saturated as the last step. (D) The column is filled with dry soil and saturated. A larger portion is excavated and the seed placed into the hole. The hole is filled again with the previously excavated soil and compressed by tapping. (E) The soil storage is pre-wetted and the column packed with this soil in two layers placing the seed in between the layers.

Figure 1. Packing methods used for the determination of a realistic field condition. Each method was set up using three replicates of a pelleted and coated seed. (A) The column is filled with dry soil, saturated and the seed placed centrally on the layer. A second layer is poured onto the seed and the capillarity force saturates the second layer. (B) The column is filled completely with dry soil and saturated. A hole in the size of the seed is dug and the seed placed into the hole. The hole is filled with dry soil and capillarity force saturates the filling. (C) A first layer is packed dry, the seed sown on top the layer and a second layer applied dry as well. The whole column is saturated as the last step. (D) The column is filled with dry soil and saturated. A larger portion is excavated and the seed placed into the hole. The hole is filled again with the previously excavated soil and compressed by tapping. (E) The soil storage is pre-wetted and the column packed with this soil in two layers placing the seed in between the layers.
resolution while not influencing the germination and establishment of the sugar beet seeds, a trade-off well known in CT. The radicle angle was very close to 90° in all samples and therefore the radicle was not touching the column wall and the radicle lengths did not at any point exceed the length of the column.

Columns were scanned daily using a Phoenix v|x m 240 kV (GE Measurement & Control Solutions, Wunstorf, Germany). A potential energy of 130 kV with a current of 100 μA and a lasting of 250 ms was applied collecting 2878 angular projection images in constant rotation mode (FAST SCAN), where image average and skip values were set to 1 and 0, respectively. Scans were performed at a spatial resolution of 20 μm with an acquisition time of 12 min each in a multi scan acquiring two sections. Acquisition images were reconstructed using the phoenix datas|x 2rec reconstruction tool (beam hardening was set to 8, region of interest and scan optimisation has been automatically calculated) resulting in 16 bit data. The soil columns were scanned in the same order every day to reduce the impact of temporal influences and create a 24 h difference between each scan. However, the germination was initiated at the same time for the whole sample set to raise the seedlings in the same day and night rhythm. Therefore there was difference of about 6 h between the first and the last scan of the day, however this had a negligible effect on the data interpretation as the shift in radicle length between replicate 1 and replicate 4 of each treatment was minimal and was mostly accounted for by natural variation which can be extrapolated to the 6 h time difference.

Data processing was performed using VGStudio Max® 2.2. Seedlings were segmented using the 3D region growing tool and root lengths determined using the polyline tool as described by Tracy et al (2012). The average thickness of the coating and pelleting was manually determined by using the distance tool on multiple sections of the seed in different 2D view orientations. The soil to air ratio for the different packing methods was determined by segmenting the seed as a solid object without inner air space and dilating the segmented area by 1 voxel (20 μm). The segmented seed was subtracted from the dilated seed so that a ring of 1 voxel thickness remained. A surface determination based on air space as background and several areas of soil as material was used to determine soil and air space volume which was used to calculate a percentage.

An additional destructive screening experiment was conducted to support the work with increased replication (20 per treatment). Half of the corresponding seedlings were excavated after two days of growth, the other half after four days. The excavated roots were washed and the root lengths determined using graph paper. All error calculations have been conducted using the standard error of the mean.

Results

Method development

Preliminary investigations were undertaken to assess the appropriate soil packing method (five in total) to create realistic field conditions (figure 1). Figure 2(A) shows the capillary method was responsible for the formation of two distinct layers. The bottom layer consisted of a higher percentage of fine particles at the transition zone, whereas the top layer showed a higher amount of coarser particles which resulted in a hydraulic disconnection causing the developing root and stem to push the top soil layer upwards. A ratio of 55.76% (±4.56) soil to 44.24% (±4.56) air was calculated within a distance of 1 voxel (20 μm) around the seed. Method B led to a higher seed–soil contact around the seed. Nevertheless, the filled region featured more pore space than the surrounding soil than would be considered ideal (figure 2(B)). Method B showed the highest surrounding soil mass with a ratio of 68.86% (±2.09) soil to 31.14% (±2.09) air. Method C exhibited a high seed–soil contact throughout the whole region with the most uniform distribution of the fine soil particles with a ratio of 63.01% (±0.83) soil to 36.99% (±0.83) air (figure 2(C)). Excavation of soil to insert the seed resulted in large air space pockets around the seed although the top layer was tapped downwards (figure 2(D)). These air pockets resulted in a higher amount of air space around the seed (38.49% (±5.94) soil to 61.51% (±5.94) air). A similar appearance was observed in method E with a ratio of 32.86% (±12.31) soil to 67.14% (±12.31) air (figure 2(E)). Method D and E had a significantly higher air space around the seed as well as a high variability within the replicates which was non-preferable. Method C was chosen for subsequent studies as it ensured greater reproducibility of packing and from field observations appeared to reflect a structural arrangement most similar to the field situation. However, one might expect a higher degree of heterogeneity in the field over large distances which would potentially increase variation in germination behaviour between seeds.

Comparison of seed enhancement technologies

X-ray CT scanning of a seed in air enables the distinction of different components (figure 3). The scan of the bare seed ex situ enabled measurements of the thickness of the seed coating and pelleting. The seed coating had an average thickness of 0.03 mm evenly distributed over the seed surface with a volume of approximately 1.04 mm³ (P+FC) and 1.09 mm³ (FC). Applied on the surface of a pellet seed, the thickness varied between 0.03 mm and 0.06 mm with a size of approximately 20.30 mm³ (P) or 20.72 mm³ (P+FC). The thickness of the pelleting varied highly between 0.05 mm and 1.10 mm due to the shape of the seed. The applied pelleting had a porosity of 18.03%
Figure 2. Results of the packing studies. The labelling of different methods refers to the previously explained methods of figure 1. The images have been taken from the top 2 cm of the column using the front orientation and the same scale. (A) The capillarity method resulted in the formation of a transition zone which forms a hydraulic disconnection between both layers. (B) The digging method results in a loose soil portion above the seed which is poorly connected to the surrounding soil. (C) The dry method results in uniform distribution of soil particles around the seed. (D) The excavation method results in the appearance of air pockets around the seed. (E) The field moist method results in highly disconnected soil portions in the column.

Figure 3. 2D images of non-germinated sugar beet seeds. (A) Naked seed. (B) Coated seed. (C) Pelleted seed. (D) Pelleted and coated seed. This scan was taken with a resolution of 5 μm.

for P and 15.82% for P+FC. Figure 4(A) shows an exemplar 2D image slice of an x-ray CT scan of a 3 day old seedling in soil. The 20 μm resolution enabled a differentiation of the fruit wall, the perisperm and the embryo for the first time in soil. The shoot can be observed growing towards the soil surface and thereby
pushing the soil particles aside. Figure 4(B) shows a similar appearance for treatment P + FC. The formation of the apical hook results in a region of higher compaction in comparison to the soil particles around the seed. It appears that the number of small soil particles in immediate contact with the seed is much higher in comparison to larger particles which can be precisely observed using x-ray CT. The grey value intensity differences observed in the pelleting highlight the layers consisting of materials with different x-ray attenuation coefficients where the lightest parts represent mineral based components. On the outermost layer is a fine white line (high x-ray absorption) which is due to the mineral content of the pesticide coating.

Quantitative assessment of x-ray CT data

Sugar beet radical growth characteristics for each seed treatment were measured daily for four days (figure 5). Treatment N was shown to display a rapid growth response followed by two days of steady growth (figure 6(A)) whereas P + FC and P had an initial slower growth that increased over the four days resulting in longer radicle length of P compared to N after four days of growth. Specifically, the radicle lengths of P + FC were ca 50% less in comparison to N on day 2 but showed a similar length at day 4. FC displayed a delay in root growth of ca one day and a slower initial development compared to the P + FC. This was followed by a rapid growth between day 3 and 4 but it did not subsequently achieve the same growth as the other treatments. The effect on the growth per day can be seen in figure 6(B).

Comparison of daily radicle growth (figure 6(B)) showed a rapid growth at day 2 for N and P which decreased the following day. The P + FC seeds showed a continuous increase in radicle growth, whereas N and P decreased after the initial rapid growth. For FC almost no radicle growth was visible at day 2 but a
Figure 5. Exemplar temporal representation of 3D reconstructions of a naked sugar beet seed. The scans were taken at a resolution of 20 μm.

Figure 6. Analysis of radicle growth based on a growth period of four days. (A) Radicle length comparison over 4 days. (B) Radicle growth per day. Radicle growth per day is calculated as the subtraction of two consecutive days. (C) Radicle volume change over time. Radicle volume is calculated automatically using VGStudio Max 2.2. N = 3, error bars are calculated using standard error of the mean.
rapid increase in growth occurred on the subsequent days.

A similar pattern was observed for the radicle volume between all treatments (figure 6(C)). The volume determination suffers from an intrinsic potential error of up to 18%–20% for a dilation by 1 voxel (addition of a one voxel layer) and up to 16%–18% for an erosion of 1 voxel (subtraction of a one voxel layer) for the majority of the segmentations. N and P treatments showed a rapid increase in volume at day 2. The growth rate extenuated at day 2 but accelerated again at day 4. P+FC treatments showed an almost linear increase in radicle volume. A delay was observed for FC with a rapid acceleration on the last day. Figure 7 displays the ratio of radicle volume and radicle length to visualise the differences between the seed enhancement methods. There was a significant relationship interaction between sampling day and seed treatment (p = 0.004). A screening test was conducted to observe the variability within each treatment type (displayed in brackets is standard error): Day 2: N 8.4 mm (±2.4 mm), FC 0.2 mm (±0.2 mm), P 6.1 mm (±1.3 mm), P+FC 4.2 mm (±1.3 mm); Day 4: N 35.6 mm (3.5 mm), FC 18.7 mm (±5.2 mm), P 38.5 mm (±4.1 mm), P+FC 38.7 mm (±0.9 mm).

A comparison of the x-ray CT data and the screening data was conducted (figure 8). The radicle lengths measured using the x-ray CT data were higher compared to the screening data in all treatments and both screening days.

Discussion

This study successfully highlighted subtle temporal differences in growth between different seed enhancement treatments using x-ray CT and hence verifying x-ray CT as a suitable tool for the quantification of the establishment process of plants i.e. our work was undertaken on sugar beet but is transferable to most seed types. Due to the nature of the in situ environment, the contrast of the collected images suffers in comparison to a scan of a seed outside of soil which is a limiting factor for observing the germination process.
in the seed, though we believe this is offset by the advantages of observing behaviour in soil. The results show for the first time in soil clear treatment differences in radicle growth characteristics over the first four days after sowing. Although the results generally showed low within treatment variability, an additional screening test of ten replicates was used to further understand the inherent variability of seedling establishment. Results indicated that the radicle length of all seed enhancement types have a high variability. It was noticed, however, that radicle lengths were higher when measured by x-ray CT compared to excavation measurement. It has previously been reported that radicle lengths can be underestimated using x-ray CT which contradicts these findings (Mooney et al 2012). However, in this study different plants were used for the x-ray CT and the destructive analysis so we attribute natural variation as the main reason for the difference. As the length differences were significant throughout all treatments, it may be possible that x-ray radiation had a small but beneficial effect in the small doses that are able to penetrate the soil (Shull andMitchell 1933). A further beneficial effect might be due to an inhibitory effect on pests that could be present in the soil or the seed itself (Ikram et al 2015) although this is less likely in the timescales of this study.

The use of FC seeds verified the assumption that the chemical coating, at least when applied directly to the seed, can inhibit early growth of the radicle. It has been reported that the pelleting serves to increase the spatial distance to the coating besides its original purpose to increase the ease of planting (Kaufmann 1991, Hill 1999, Taylor et al 2001). The application of pelleting resulted in a higher germination rate compared to other treatments based on root length growth per day which might be due to the increased water uptake rate as a beneficial side effect of the pelleting material consisting mainly of wood fibre and clay. The growth over time showed a very rapid development of the radicle on day 2 for all treatments except for FC. The fast radicle growth for the FC treatment started with a day delay which is likely due to the proximity to the insecticides and fungicides. Standard pesticides used for sugar beet protection include fungicides like Thiram or Tachigaren (active ingredient: Hymexazol) as well as insecticides like thiamethoxam, imidacloprid or chlothiondin (Syngenta 2016a, 2016b, KWS 2017). Redfearn and Osborne (1997) showed a quicker emergence using Thiram compared to previous seed coating treatments with similar effectiveness. Hymexazol has been reported to affect fungal RNA and DNA synthesis and should the transformed into glucosides with fungi toxic effects as well as plant growth promoting effects rapidly after entering a plant organism (Ypema 2003). Since the 1990s, neonicotinoids (e.g. thiamethoxan) have been widely used to reduce the risk of virus yellows and to control foliar and soil pests (Bayer 2011, Syngenta 2016c, KWS 2017). Though neonicotinoids, in particular, are controversial due to the reported effects on bee populations (Rundlöf et al 2015, BBRO 2016). The addition of a coating reduces the ability of the seed to open as quickly as a naked seed. The delay for FC could therefore be due to the physical shell that was created during the coating process or the resulting phytotoxicity posed by its proximity to the seed surface. Similar effects have been reported for oil seed rape as imidacloprid and thiamethoxam suppressed root system development in the cotyledon stage (Huang et al 2015). Vin and Murua (2001) found uncoated seeds develop earlier than coated seeds, as found in this study. This reduction in growth was observed only in FC and not in P+FC which may be due to the composition of the pelleting material or to the swelling of the pellet during water uptake which might help to overcome the physical shell of the coating by weakening the structure of the shell. It is likely that the slower germination could be the influence of the coating material, which is not as high for P+FC because the applied insecticides and fungicides are not directly in contact with the fruit (the fruit includes both the seed and the fruitwall surrounding the seed) and therefore the phytotoxicity impacting the seed is lower. Furthermore, a seed establishment delay was observed in different coated turfgrass species using different irrigation techniques (Serena et al 2012). Kunkur (2008), however, described a higher germination rate, a higher vigour index (vigour index = (root length + shoot length) × germination percentage) and an increased field emergence in coated seeds after three months of growth. Therefore, FC seedlings may overcome this delay later during their growth and give higher field emergence compared to naked seeds based on the protection provided against negative effects like fungi or insects. The growth rate comparison showed that P and P+FC radicle growth rates increased after the initial rapid growth whereas the growth rate for N was constant. This may be due to the enhanced water uptake during the first days of growth that would have increased water storage inside the seedling in both P and P+FC, as well as an increased vigour caused by increased water uptake (Gorim and Asch 2015). A positive effect for different kind of pelletings was previously shown during early establishment especially near moisture limiting periods supporting this assumption (Scott 1975).

Figure 6 showed that there are slight differences in volume per millimetre radicle length for P and N in comparison to FC and P+FC but these were not significant. Also, the radicle volume was almost the same at day 4 which suggests seed enhancement technologies like P+FC do influence the radicle width shortly after germination in the same way as they influence radicle length. The four growth stages (seed cracking; rapid radicle growth; formation of apical hook; unfolding of apical hook) were visible in all four treatments although they were slightly delayed.
for the FC. This observation confirms that physical seed enhancement using pelleting or coating might not have altered the mechanism of emergence but rather the rate.

Conclusions

Seed enhancement technologies enable the alteration of the growth behaviour of the early seedling. Although it appeared N has the highest growth rate over the first two days, the addition of pelleting allowed a more rapid increase in root growth per day. The addition of a coating is essential to ensure a consistent yield in the field due to the addition of protection by applying insecticide and fungicide (FC). However, applying a coating reduced the seedling emergence rate which is why a pelleting in addition to the coating is used to balance this negative effect (P+FC). The positive effect of the pelleting as hypothesised was verified as was the negative effect of the close proximity of the pesticide containing coating to the seed. This study shows the benefits of x-ray imaging as a tool to compare different seed enhancement technologies in soil supporting efforts of seed breeding companies to optimise their seed enhancement compositions according to the seeds in situ performance. The physical mitigation of a pesticide coating might be overcome by priming the seed prior to pelleting which could be assessed in future studies. During priming, the germination process is initiated and stopped before the seed starts to crack. This effect is hypothesised to overcome the disadvantage of the coating delay. Future work is needed to evaluate the effects of priming in comparison to the data presented here. Additionally, the effect of different growth conditions (e.g. drought and compaction) could be monitored to gain a greater understanding of the influence of the physical treatment as well as of the priming. Factors such as these have important consequences in understanding seedling germination in a changing environment and may support sustainable agricultural practices.

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