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Application note

Computer vision under inactinic light for hypocotyl–radicle separation with a generic gravitropism-based criterion

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ABSTRACT

This article proposes a computer-vision based protocol, useful to contribute to high-throughput automated phenotyping of seedlings during elongation, the stage following germination. Radicle and hypocotyl are two essential organs which start to develop at this stage, with the hypocotyl growing towards the soil surface and the radicle exploring deeper layers for nutrient absorption. Early identification and measurement of these two organs are important to the characterization of the plant emergence and to the prognosis of the adult plant. In normal conditions, this growth process of radicle and hypocotyl takes place in the soil, in the dark. Identification and measurement of these two organs are therefore challenging, because they need to be achieved with no light that could alter normal growth conditions. We propose here an original protocol exploiting an inactinic green light, produced by a controlled LED source, coupled to a standard low-cost gray-level camera. On the resulting digital images, we devise a simple criterion based on gravitropism and amenable to direct computer implementation. The automated criterion, through comparison with the performance of human experts, is demonstrated to be efficient for the detection and separation of radicle and hypocotyl, and generic for various species of seedlings. Our protocol especially brings improvement in terms of cost reduction over the current method found in the recent literature which resorts to higher-cost passive thermal imaging to perform the same task in the dark, and that we also consider here for comparison. Our protocol connected to automation of image acquisition, can serve to improve high-throughput phenotyping equipments for analysis of seed quality and genetic variability.

1. Introduction

Seedling heterotrophic growth is a crucial stage of the development of plants. After sowing, two successive stages have to occur starting with germination until the radicle protrudes out of the seed coat and then the heterotrophic growth in the soil until the seedling emerges out of the soil. In field conditions, germination and heterotrophic seedling growth stages Taiz and Zeiger (2010) are not easily observable and diagnosis on sources of seedling emergence failure is thus difficult, especially the separation of the respective impacts of the two stages in sowing failures. Non invasive monitoring of seedling growth that would help for a better analysis of seed quality variations is accessible in laboratory conditions with computer vision machines A common imaging system, reported in Jaffe et al. (1985), Ishikawa and Evans (1997), Walter et al. (2002), Kimura and Yamasaki (2003), Wang et al. (2009a), French et al. (2009), Belin et al. (2011), and Subramanian et al. (2013) with various levels of automation, consists in monitoring a set of seedlings positioned on a row in a vertically settled box with agar gel. A backlight system associated with a camera then produces sequences of images of seedling during growth. From such image sequences, the temporal evolution of the length of the seedling is measurable with classical image processing such as binary image skeletonisation. The studies, made possible by such computer vision systems, target various biological processes during autotrophic growth and including plant gravitropism (Jaffe et al., 1985; Ishikawa and Evans, 1997; Subramanian et al., 2013), hypocotyl elongation in controlled light and temperature

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conditions (Walter et al., 2002), root length and diameter measurements (Kimura and Yamasaki, 2003; French et al., 2009), photomorphogenesis (Wang et al., 2009a). When used during the heterotrophic growth, a major limitation of these computer vision tools is the need for light in the image acquisition step. Normally, the seedling grows in the soil in the dark, and if exposed to light, its upper parts stop elongating because of the seedling light receptors, and new leaves expand. Therefore, if light is used to monitor heterotrophic growth of seedling, it has to mimic obscurity for plant, i.e. to be non "perceived" or absorbed by plant cells. We define such a light as inactinic. Some authors use infrared LED to work in absence of light visible to human eye and also because it is known in the autotrophic growth that the leaves of the plants are sensitive to the contrast between red and infrared light (Smith, 2000). This strategy however is not suited to mimic obscurity for plant during heterotrophic growth. Recently, it has been shown that seedling elongation was accessible with computer vision in complete absence of light, thanks to the use of passive thermal imaging (Belin et al., 2011, 2014). From thermal contrasts, Belin et al. (2011) shows that it is possible to segment the seedling in the dark and also that it is possible to discriminate in the seedling two sub parts: the radicle and the hypocotyl. Following a gravitropic response, the upper part of the seedling, the hypocotyl, grows to reach the light and activates photosynthesis, while the lower part of the seedling, the radicle, grows deeper to anchor in the soil and provide access to water and nutrients. It is important for plant phenotyping to identify hypocotyl and radicle early after their formation in the seedling, and to follow their development during seedling elongation. Such observations carry useful relevance for the better understanding of variations in plant emergence. The elongation rates of the radicle and the hypocotyl have been demonstrated as key input parameters to predict crop emergence in the soil in various models, e.g. Forcella et al. (2000), Dürr et al. (2001), and Brunel-Muguet et al. (2011). The separation of radicle and hypocotyl obtained from thermal contrasts established in Belin et al. (2011) can be used as a reference for very precise measurements, but there are limitations in the use of thermal imaging to monitor seedling growth which are the high cost of thermal imaging and the rather limited spatial resolution of thermal cameras impacting the throughput of such phenotyping systems.

In this study, we address the separation of the hypocotyl and radicle of seedling in elongation with a high resolution imaging system working with inactinic lighting conditions in the visible spectrum. We consider a green LED technology for the imaging system and propose to investigate the inactinic properties of such lighting. We introduce an image processing algorithm based on the prior information of gravitropism to separate radicle and hypocotyl in these lighting conditions, and demonstrate results in accordance with the thermal contrasts and with the visual appreciation by human experts. The computer vision tool presented is shown generically efficient for various seedling species.

2. Materials and methods

2.1. Imaging systems

We use two different imaging systems. The main imaging system is an original system we propose with inactinic green light and gravitropism-based image processing criterion to distinguish two organs: radicle and hypocotyl. The second imaging system based on thermal imaging is only used to compare results obtained with main imaging system according to the distinction between organs. The two different imaging systems are acquiring images at a sampling time period of 2 h during time periods of 50 to 100 h following the sowing.

Main imaging system: A standard 8 bits 640 × 480 pixels gray-level monochrome camera is used. The image acquisition by this gray-level camera is synchronized with an on/off switch of a light. Plants have light sensors, called phytochromes and cryptochromes, which are sensitive either to red/far red or blue wavelengths expositions respectively (Smith et al., 1990; Smith, 2000; Briggs and Olney, 2001). A method commonly used for mimicking darkness for plants is therefore to observe them under green light, as the plant photoreceptors are assumed not to be activated under wavelengths in the range (515–550) nm. In our experiment we used a light realized with green LED with spectrum in the range (515–550) nm. For the segmentation of radicle and hypocotyl, the seedlings are placed vertically in a box with agar gel so as to let the seedlings follow the natural gravitropism during their elongation. The agar gel makeup is in proportion of 9g of Agar HP 696 KALYS for 1l of nutritive solution prepared as described in Saglio and Pradet (1980). The agar gel brings the water nutrient to the seedlings, and serves as mechanical support to prevent the seedlings from falling during their elongation. The green light and gray-level monochrome camera are arranged on a backlight mode (the box

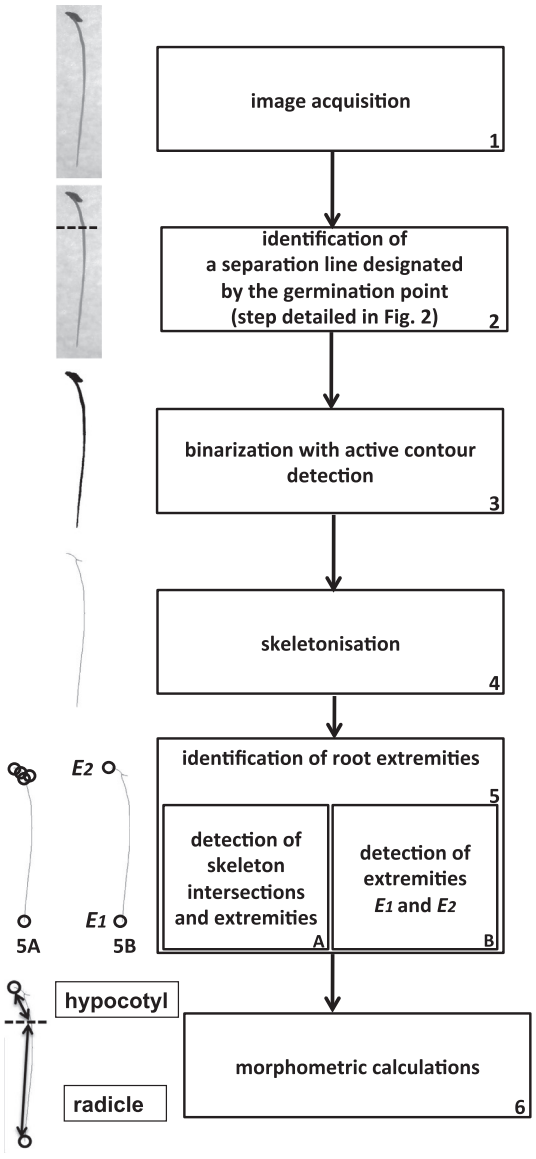

Fig. 1. 6-step algorithm for automated image processing of radicle–hypocotyl separation with the visible imaging system.

with agar gel is placed between the camera and the LED) and are switched on in synchronization with image capture. The entire system is placed in a growth chamber regulated at 20 °C.

Imaging system 2: for comparison with results obtained in visible spectrum with the main imaging system for the hypocotyl–radicle separation, we use the thermal imaging system described in detail in Belin et al. (2011) that we briefly recall here. Seedlings are placed on a wet blotter in a compartment regulated in temperature at 20 °C. The compartment is equipped with a sliding cover, opening in synchronization with thermal image acquisition by a FLIR SC5000 cooled infrared camera with a spectral range between 2.5 and 5 μm, a spatial resolution of 320 × 240 pixels, and a 12-bits dynamic, positioned in front of the compartment. A standard 8 bits 640 × 480 pixels gray-level monochrome camera is also attached to the thermal camera so as to share almost the same optical axis. The resulting imaging system produces pairs of gray-level intensity images and thermal images of the seedlings placed in the compartment. The angle between the two optical axes of the cameras being very small, the two images are easily registered with a rigid rotation–translation registration algorithm (Goshtasby, 1988).

2.2. Image processing algorithm

The segmentation of the radicle and hypocotyl organs of the seedlings has been automated following a 6-step algorithm depicted in Fig. 1. Step 1 is the acquisition of visible images of seedlings positioned vertically. Step 2 corresponds to estimation of the separation line from an automated identification of the germination point as introduced in Ducournau et al. (2004) and

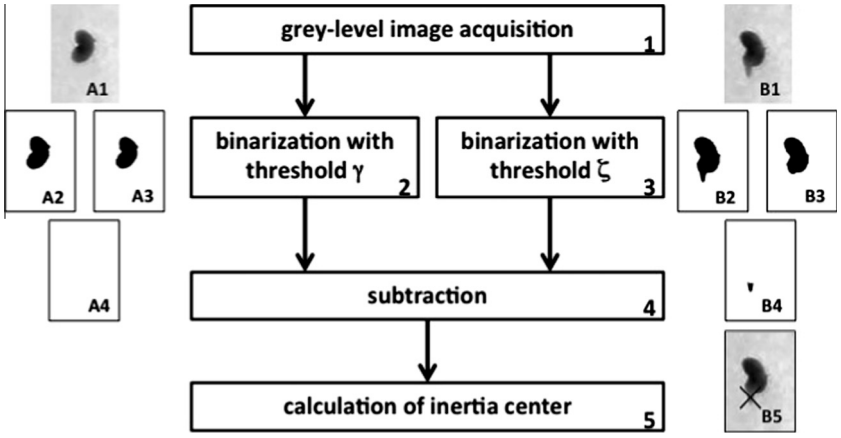

Fig. 2. A1 is an image of a seed before radicle protrusion and B1 after radicle protrusion. The first binarization with threshold γ separates seed (A2) and seedling (B2) from background. The second binarization with threshold ζ only detects the dark part of seed (A3) and seedling (B3). The subtraction of these 2 Images respectively (A2, A3) and (B2, B3), displayed in A4 and B4, allows to detect the radicle. The cross in B5 represents the inertia center of the radicle. Thresholds (γ, ζ) are experimentally fixed and depend on species and conditions of image acquisition as described in Ducournau et al. (2004).

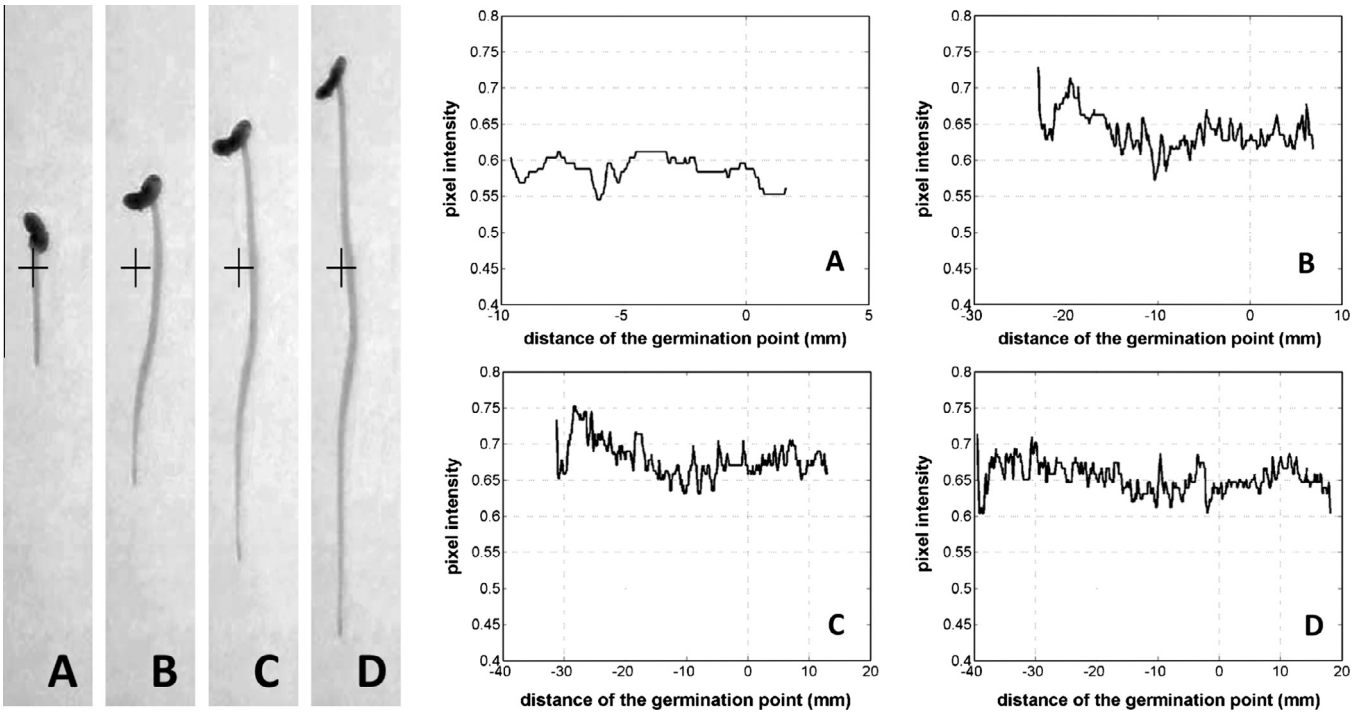

Fig. 3. Four gray-level images of one seedling during elongation (A: 40h, B: 64h, C: 76h, D: 88h). On the left, black crosses indicate the position of the germination point indicating the horizontal row of the separation between hypocotyl and radicle. On the right, evolution of the gray-level of pixels along the skeleton of the seedling with abscis zero at position of the germination point.

implemented in Joosen et al. (2010). Shortly, as illustrated in Fig. 2, time lapse images of germinating seeds are binarized according to two thresholds and the subtraction of consecutive binary images are computed to produce a change detector. The germination point is measured as the inertia center in the first subtraction image which contains non zero values. Following gravitropism, the radicle is expected to grow down while the hypocotyl raises upwards. The germination point is therefore proposed as a simple indicator of the separation between radicle and hypocotyl during seedling growth. The use of germination point as reference to separate radicle and hypocotyl is also proposed because as shown in Fig. 3, there are no intensity nor morphological contrasts between radicle and hypocotyl in the gray-level intensity images acquired in the visible spectrum. Step 3 corresponds to a binarization of the images in order to separate object pixels from background pixels and give a mask of seedling. This binarization is obtained after segmentation by a classical active contour method (Chan and Vese, 2001). Step 4 realizes a skeletonisation (Kong and Rosenfeld, 1996) on the seedling mask (extracted from the binary image). Step 5 performs an identification of root extremities. This step is in 2 stages (5A then 5B). Stage 5A detects all intersections and extremities of the skeleton. Stage 5B selects the extremities E1 and E2 which correspond respectively to the lowest point of the radicle and the highest point of the hypocotyl. Step 6 calculates morphometric parameters on the skeleton of step 4 by evaluating curve lengths for radicle and hypocotyl, both delimited with the separation established from the detection of germination point. In this study, we focus on two morphometric parameters, the curve length of the hypocotyl and the curve length of the radicle.

Table 1
Average hypocotyl length (and standard deviation) when submitted to green light or dark conditions.

Hypocotyl length (mm) after	Green light (mm)	Dark conditions (mm)
4 days	34.5 ± 11.6	32.1 ± 13.8
10 days	66.9 ± 17.3	80.4 ± 13.3

2.3. Tested seeds

To investigate the inactinic effect of green LED light, a set of 30 seedlings of *Medicago truncatula* are sown in pots of sand incubated in the dark at 20 °C. Among the 30 seedlings, 15 seedlings are exposed to green light during pulses of 15 min per day. These 15 min pulses of green light are useful to realize non destructive measurements of hypocotyl length with a small ruler applied along the seedling. At the end of the experiments, seedlings are taken out of the pots; the length of hypocotyl not visible in the sand is measured and added to all the lengths previously measured on the same seedlings. Destructive measurements are also performed 4 and 10 days after sowing for seedlings grown in complete dark

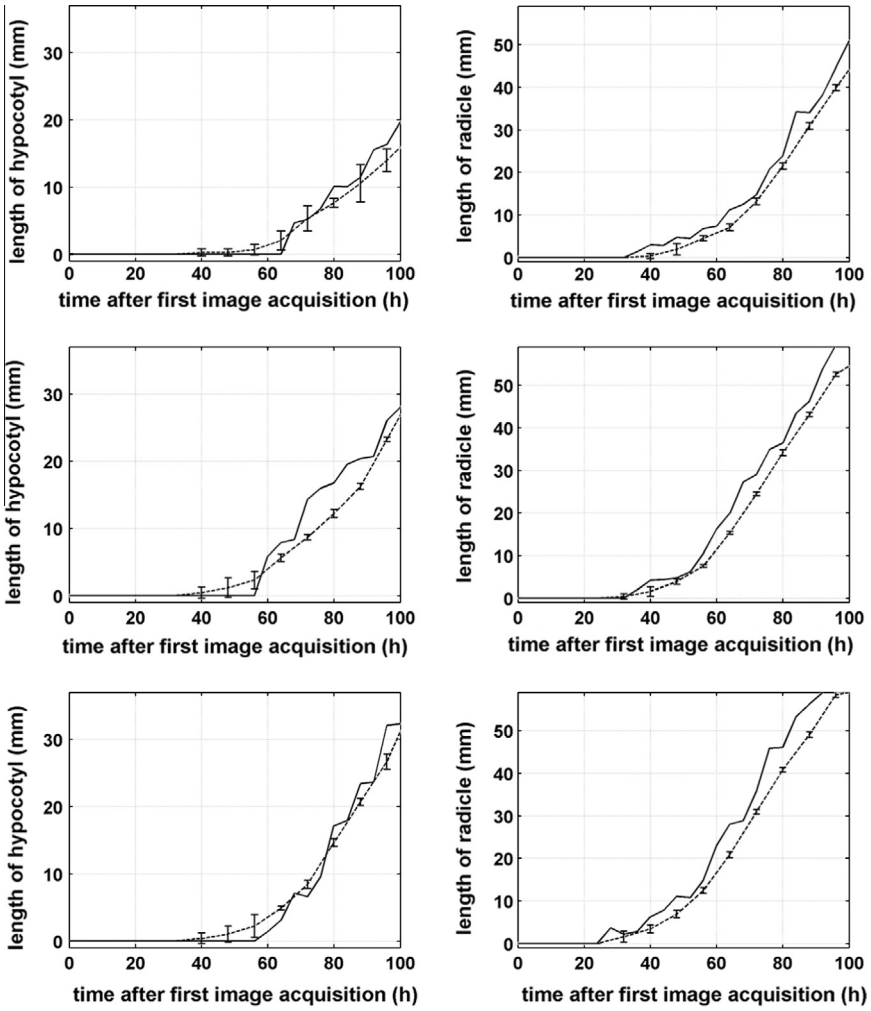

Fig. 4. Hypocotyl (left column) and radicle (right column) length of three seedlings of *Medicago truncatula* as a function of time. Solid line is from automated measurements from visible imaging system and dashed line is the average and associated standard deviation with error bars for the manual measurement from 4 experts. Each row corresponds to one seedling.

conditions. To evaluate the inactinic effect, we manually measure the length of hypocotyl, which is the only organ of seedlings having light sensitive phytochrome (Smith and Whitelam, 1990).

M. truncatula is used in the comparison between results of automated processing of the visible imaging system, thermal imaging and reference performed by experts, as it was already chosen in Belin et al. (2011) for comparison between thermal imaging and the visual appreciation by an expert. *Arabidopsis Thaliana*, another important plant model in plant science was not adapted in this context due to limitation of the spatial resolution of the thermal imaging system. In this study, we also extend to seedlings of agronomic interest including oil rape, sugar beet and wheat. These species have been added in the experiment done with the main imaging system working in the visible spectrum. It is important to note that the same segmentation algorithm has been used on these four species.

2.4. Ground truth comparison

The segmentation of the radicle and hypocotyl realized from images acquired by our main imaging system are manually measured by four experts. Their measurements can be considered as reference or ground truth with an uncertainty taken as the standard deviation between the four estimates given independently by the four experts.

3. Results and discussion

3.1. Inactinic properties of green LED lighting

We first demonstrate the inactinic properties of green LED light. As visible in Table 1, the study, realized on 30 seedlings of *M. truncatula*, shows no significant effect of green LED on seedlings development by comparison with development in darkness. This is specially true after 4 days which approximately corresponds to the duration of the monitoring presented in this article.

3.2. Validation of automated measurements by confrontation with experts notation

Since no impact of the green LED light is observed on seedlings development, we now assess the image processing algorithms described in Fig. 1. We first present the confrontation of automated measurements with the manual segmentation of the experts. The results, displayed in Fig. 4, show a good agreement between ground truth and automated measurements which fall in the interval of uncertainty of the manual ground truth measurement obtained from the four experts. This constitutes a first validation of the germination point as a simple efficient criterion to distinguish radicle and hypocotyl from a simple imaging system working in the visible spectrum. To further validate this criterion, we tested this imaging system with other species, namely oil rape, colza, wheat and sugar beet at various elongation stages. We performed the comparison between ground truth and automated measurements on 400 images. On this data-set, our segmentation method presents a bias of 5% overestimation of the hypocotyl length. Skeletonisation, step 4 in the image processing chart flow of Fig. 1, is known to be sensitive to noise which can produce extra small branches increasing the measured length of the skeleton. It is common practice to apply skeleton de-noising to remove branches of length non compatible with prior knowledge on seedling anatomy. We could, in further studies, apply such de-noising techniques to reduce the observed bias but it is first important to note the good quality of the basic measurement realized here given the simplicity of the image processing chart flow used in this study.

3.3. Validation of automated measurements by confrontation with thermal imaging system

To extend the demonstration of the validity and robustness of our radicle-hypocotyl separation criterion, we now compare with results obtained from thermal imaging considered as objective tool for the separation and the measurements of organs along seedlings

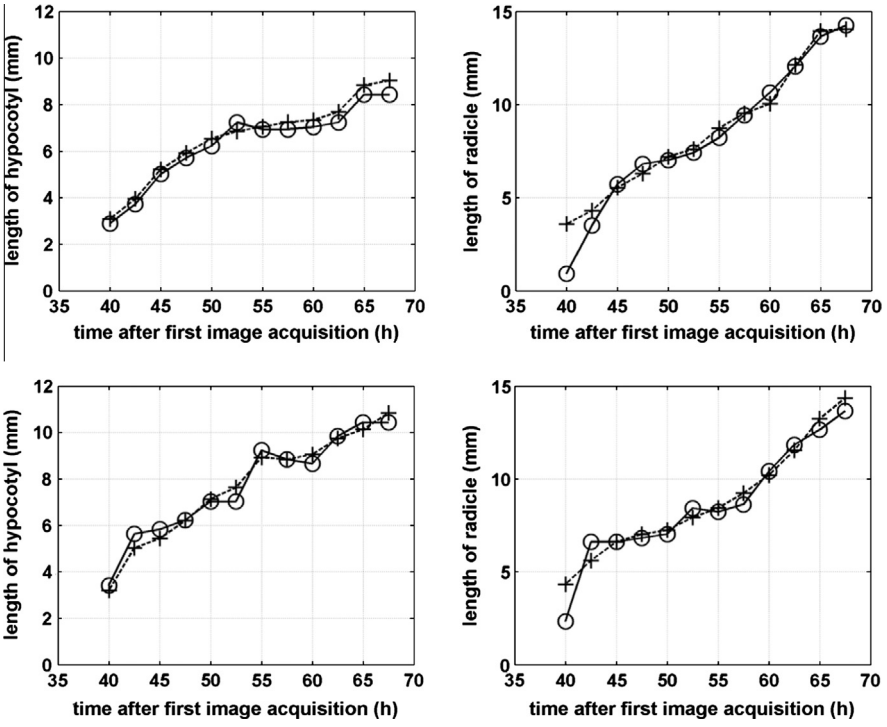

Fig. 5. Hypocotyl (left column) and radicle (right column) length measurements from thermal and corresponding visible images. Solid lines are automated measurements on thermal images obtained from the algorithm of Belin et al. (2011). Dashed lines are the measurements on the corresponding visible images with the automated algorithm of Fig. 1. Each row corresponds to one seedling of *Medicago truncatula*.

(Belin et al., 2011, 2014). The separation of radicle and hypocotyl on thermal images is determined from thermal contrast between radicle and hypocotyl (see Belin et al. (2011) for a detailed description), i.e. a criterion different from the gravitropic criterion used with the visible imaging system. The result of the confrontation between these two independent imaging systems is displayed in Fig. 5. This demonstrates that the separation between radicle and hypocotyl detected from thermal contrasts with thermal imaging and from the gravitropic criterion with the visible imaging system are in good agreement. The relative standard deviation of length produced by these two techniques and averaged over the 50 pairs of images acquired for Fig. 5 is equal to 3.01% in the hypocotyl and to 1.75% in the radicle. This constitutes an objective validation of the gravitropic criterion proposed in this article.

4. Conclusion

For the automated phenotyping of seedling by computer vision the separation between hypocotyl and radicle is an important step not routinely handled by standard image analysis systems. To contribute on this important phenotyping task, we have proposed here a green LED imaging system associated with a simple criterion based on the recording of the germination point. We have tested the validity and robustness of this criterion by performing hypocotyl–radicle separation on computer images from seedlings of different species. This has been assessed by comparison with human expert and with computer vision based on another independent criteria from thermal imaging.

This result is important since it is established with a system working with gray-level low-cost imaging and green light. Such a low-cost visible imaging system can now serve as reference in place of thermal imaging, which proved capable of separating with high precision hypocotyl and radicle but with a much higher-cost imaging system.

In this study we focus on the separation between hypocotyl and radicle which is an important phenotyping step. There is a third part in the seedling which are the cotyledons. The measurement of these cotyledons is important as their size influences the initial size of the first photosynthesis leaves after seedling emergence out of the soil (Boiffin et al., 1992). Their size is correlated to the initial seedling size after emergence and this was included into models that predict growth after emergence (Dürr et al., 2001; Fayaud et al., 2014). As shown in Fig. 3, the contrast in gray level between cotyledons and hypocotyl is much higher than between radicle and hypocotyl. A raw segmentation of hypocotyl and cotyledons therefore can appear as an easy task compared to the one tackled in this report. However, determining the very beginning of the hypocotyl is shape dependant. Cotyledons segmentation has been tackled so far for plant such as the dicotyledon species *Arabidopsis Thaliana* (Wang et al., 2009b) with morphometric criterion. The adaptation of the segmentation strategy for this plant remains uncertain for other species having different shapes of dicotyledons and it would therefore be interesting to consider if generic simple criteria like the one proposed in this study could be unraveled for this segmentation task. Moreover, the procedure should also be tested for monocotyledon species as they still present another shoot shape.

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