Integrating Multiple-capsule Traits Quantitative Evaluation of Seed Maturity by 3D Phenotypic Platform in *Nicotiana tabacum*

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Abstract. Many methods have been proposed for the identification of seed maturity, and almost all of them need to be performed after seed harvest. In this study, a real-time quantitative method that can be performed during seed development was used by integrating multiple-capsule traits using a high-throughput screening (HTS) technique. Capsule color, shape, and density parameters can reflect seed development and maturity. During seed development, we observed a fast decrease in color parameters (R, G, and B) and water content, as well as an increase in temperature sensitivity; an initial rise followed by decline in shape parameters [length, width, minimum circumscribed circle (MCC) diameter, area] was also observed; as well as irregular differentiation of density parameters of the capsules. Correlation analysis showed a significant relationship between seed maturity and capsule color, as well as its shape parameters (Table 1). In sum, our data demonstrate that that three-dimensional (3D) phenotypic platform can be used to differentiate seed maturity by quantitative evaluating multiple-capsule traits, which is a quantitative method for determining the maturity of seed while still growing in the fruit of the mother plant.

Seed development is normally divided into three stages: morphogenesis, reserve accumulation, and maturation drying (Kermode et al., 1985). At the morphogenetic stage, the seeds do not possess germination ability, which is gradually obtained with the accumulation of reserve. Seed vigor gradually increases after physiological maturity, whereas seed desiccation is not only

drying of the seed but also an active stage of preparing for germination (Angelovic et al., 2010). Numerous studies have confirmed that the seed maturity has a strong influence on its vigor (Hay et al., 1997; Jalink et al., 1998). Physiological maturity of the seed (Ellis and Pieta Filho, 1992), and maximum accumulation of dry seed weight represent maximum seed yield in many crops and have also been proposed to represent maximum seed vigor.

An image analyzing system for seed vigor was first used by McCormac et al. (1990). They captured a seedling image of lettuce with a video camera and then measured the root length on the image by hand, which was a revolutionary attempt to determine seed vigor. Currently, there are various image analysis methods for seed testing, such as vigor tests (Howarth and Stanwood, 1993), purity analysis (Chtioui et al., 1997), or physical characterization of seeds (Anouar et al., 2001; Kruse, 2000). Sako et al. (2001, 2004) developed

a computer-controlled system for seedling image analysis and seed vigor testing that integrates image acquisition and processing technology. However, the evaluation of seed maturity using fruit images is rarely employed and is a more valuable approach for seed production.

Capsule color has been used for evaluation of seed maturity in tobacco for a long time. Accordingly, based on color, capsule goes through three developing stages: green ripening, yellow ripening, and brown ripening. Nevertheless, this is a subjective approach, and it is difficult to ensure that the harvested seeds are of the same maturity. Therefore, an objective testing method on seed maturity that can be completed in the field is of utmost importance. Previously, we demonstrated that CIELAB color space is a quantitative method for capsule color measurement, which could be used to imitate the seed development and predict seed vigor in tobacco (Li et al., 2015a). In addition, CIELAB color space is a useful tool for analyzing the dryness of the capsules and determining harvest time (Li et al., 2015b). However, misinterpretations may occur because CIELAB color space is only a single color indicator. Therefore, the objective of this study was to develop a new method that could integrate multiple capsule traits for assessing capsule development and prediction of seed maturity in tobacco. This approach has two aspects: First, it is necessary to establish whether the 3D phenotypic platform can differentiate capsule maturity through quantitative evaluation of traits, such as capsule color, shape, density, water content, and temperature sensitivity. Second, if difference in those parameters among physiological developmental capsules is observed, can this be clearly identified through mechanical sensing?

Materials and Methods

Plant cultivation and capsule development

The seeds of *Nicotiana tabacum* L. ‘Yunyan 85’ were obtained from Tobacco Institute in Guizhou province and were approved for use in this study. Seeds were grown in a greenhouse in Sunqiao modern agriculture garden in Pudong New District, Shanghai, China. The samples for the capsules were collected at 10, 20, 30, 40, and 50 d after pollination (DAP). All capsule samples were collected as follows: at least 45 capsules were picked from the top, middle, and bottom branches of the mother plants. Capsules were randomly selected and assigned to three groups (15 capsules per group) that were used for phenotype analysis. On the basis of biological replicates, three additional technical replicates for each biological replicate were designed.

Capsule phenotype analysis

Image acquisition. The images were acquired by a phenotypic platform Scanalyzer

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HTS (German, LemnaTec Company). The capsules were positioned in the imaging chamber, and the cameras ran across the plants on an XY grid. Over the course of project, three kinds of cameras were used: visible light (VIS), near infrared (NIR), and infrared (IR). Imaging system “Lemna Control” was used to capture the image. After imaging, it was possible to observe the image acquisition record by entering the Lemna Base and opening Snapshot Viewer. The images were derived by using the Snapshots in Lemna Base.

Image processing. For all image processing, the flexible and powerful image processing tools of LemnaTec HTS systems were used. LemnaGrid is an image analysis component of LemnaTec HTS systems. The analysis process included five steps: 1) image preprocessing (demonstrating, foreground, and background settings); 2) image segmentation; 3) image characteristics extraction; 4) image format conversion; and 5) image post-processing. By entering the Lemna Miner and opening Query Data, it was possible to observe the data analysis and resulting data.

Phenotypic data

The software of LemnaTec HTS systems processed length, width, perimeter, area, external polygon (EP) perimeter, EP area, MCC diameter; minimum circumscribed rectangle (MCR) area, eccentricity, compactness, mean color blue, mean color green, and mean color red phenotypic data from the image acquired under visible light conditions. The relative capsule water content by NIR imaging and relative capsule temperature by IR imaging were also obtained.

Seed maturity and vigor

Radicle emergence, electrolyte leakage, and seedling robustness were used together to determine seed maturity and vigor, following previously published methods (Li et al., 2015b). The differences in the emergence rate of seedling and the fresh weights of seedling and root were measured 1 month after sowing.

Statistical analysis

The experimental design was a randomized design with three replicates. Capsule weight, color, shape, seed conductivity, and radicle traits were used for correlation analysis, and analysis of variance was performed using the Duncan's test ($P < 0.05$ error level). Statistical analyses were performed using SPSS software Ver.16.0.

Results

Seed maturity changes during development.

The 1000-grain weight and electrical conductivity of the seeds harvested from DAP10 to DAP30 were increased or decreased respectively, but the observed differences were not statistically significant.

Seeds harvested from DAP10 could not germinate either in artificial climate box or seedling greenhouse. During DAP20 to DAP40, the seed germination potential, seedling emergence rate and root fresh weight continued to increase, approaching the peak value about at DAP40. The preceding results (Fig. 1) indicate that the seeds harvested at DAP10 had no germination ability, which continuously increased during DAP20 to DAP40, and it was close to peak value at DAP40, when the seed is fully physiological maturity.

Capsule water content and distribution during seed development. During seed development, fresh and dry weight of the capsules first increased, reached the peak at about DAP20 or DAP30, respectively, and then decreased. Water content of seeds decreased rapidly from DAP10 to DAP40; however, the speed slowed down then (Fig. 2). Water distribution within a capsule was not even, the center had less moisture than the external part, especially for the DAP40 or DAP50 harvested capsules (Fig. 3C). In addition, intercomparisons were performed among the five developmental

Fig. 1. Maturity changes during seed development. DAP represents day after pollination. Thousand seed weight (TSW), electrolyte leakage (EL), germination potential, seedling emergence rate (ER), seedling fresh weight (SFW), and root fresh weight of seedling (RFW) were used together to determine seed maturity (means ± SD).

Fig. 2. The change of capsule water content during seed development. DAP represents day after pollination (means ± SD).

stages of the capsules, which showed dependence: the more mature seeds were, the lower their water content was. Correlation analysis also showed the significant relationships between water content and color parameters of the development capsules.

Capsule color changes during seed development. During seed development, the capsule color changes from the initial green to yellow and then brown (Fig. 3A). The CIELAB color space indicated a decrease in L^* , b^* , C^*_{ab} , and h_{ab} parameters and an increase in a^* were observed (Fig. 4A). The visual light of the 3D phenotypic platform showed a progressive decrease in R, G, and B parameters (Fig. 4B). This can be interpreted as a decline in yellow and green light, and a rise in red and blue, which results in brownish capsule color.

Capsule shape changes during seed development. During seed development, the capsule size gradually increased from DAP10 to DAP30, and then progressively decreased from DAP30 to DAP50 by sensory evaluation (Fig. 3A). Length, MCC diameter, MCR area, and eccentricity ratio of capsule traits displayed almost similar variation (Fig. 5). Correlation analysis showed significant relationships among capsule shape parameters of width, perimeter, area, EP area, and EP perimeter with seed maturity.

Capsule temperature sensibility during seed development. Five capsules in different development stages were harvested once and then placed in a constant environmental condition, after which the capsules temperature was measured. The temperature distribution within a capsule was uneven at each developmental stage (Fig. 3B), with a similarity in the all five developmental stages: the top stage temperature was higher than that of the middle stage, which in turn was higher than that of the bottom temperature. In addition, intercomparisons were performed among the capsules harvested at five developmental stages: the more mature the capsules were, the higher the temperature was (Fig. 3B).

Discussion

During the tobacco seed development, the color of its capsule changes from green at morphogenesis to brown at maturation drying (Li et al., 2015a). The maturity of seeds has long been judged on the basis of sensory evaluations. However, people have different perceptions of the same color of the capsule, so the maturity of the seeds only evaluated by this approach alone is often inconsistent. CIELAB color space is an objective method for color measurement, which can be used to quantitatively evaluate the seed development and predict seed maturity in tobacco (Li et al., 2015a). However, misinterpretations might occur because CIELAB color space is only a single color indicator. In this study, we showed that

Fig. 3. Capsule phenotypic analysis was carried out through a 3D phenotypic platform, which contains three cameras, namely visible (VIS), infrared (IR), and near-infrared cameras (NIR). Under VIS light (A), phenotypic changes during capsule development can be quantitatively evaluated. Under IR light (B), temperature differences in different maturity capsules and different parts of the same maturity capsule can be qualitatively determined. The colors represent temperature differences, the yellower (warm color) of the color, the higher of the temperature. Under NIR light (C), water content differences in different maturity capsules and different parts of the same maturity capsule can be qualitatively determined. The colors represent moisture differences, the bluer (ocean blue) of the color stand for the higher water content.

Fig. 4. Changes of capsule color during seed development. L^* represents light, with measurement ranging between black ($L^* = 0$) and white ($L^* = 100$). The coordinate a^* is positive for red colors and negative for green, whereas b^* is positive for yellow colors and negative for blue. The coordinate h_{ab} is a qualitative attribute of color, whereas C^*_{ab} is considered the quantitative property of colorfulness and allows assessment of the degree of difference in any given hue relative to a gray color with the same lightness. B, G, and R represent the blue, green, and red color, respectively. MBV, MGv, and MRV represent variance coefficient of blue, green, and red color, respectively (means $\pm$ SD).

3D phenotypic platform could be used to quantitatively measure capsule color by integrating multiple-capsule traits. Nevertheless, to use 3D phenotypic platform for color

evaluation, three RGB parameters need to be used together, unlike CIELAB color space, where a single parameter representing a color is enough. For example, a^* is

positive for red colors and negative for green, whereas b^* is positive for yellow colors and negative for blue (Yam and Papadakis, 2004). Accordingly, it appears that the color system for 3D phenotypic platform needs to be upgraded, to become more intuitive and even consistent with human sensory evaluation.

The application of image processing technology in agricultural engineering begun in the 1980s. The target image was captured first and then denoised, enhanced, recovered, segmented and processed otherwise. Finally, the phenotype parameters of the target object are used to complete the measurement and analysis (Li et al., 2014). These image-analyzing technologies have been used for seed testing (Anouar et al., 2001; Kruse, 2000; McCormac et al., 1990; Hoffmaster et al., 2005; Sako et al., 2001). However, they are still not accurate enough to measure capsule development, mainly due to insufficient accuracy. Recently,

the technology based on stereo vision and high precision camera with three-dimensional spatial information has gained popularity in plant morphology measurements (Chénét et al., 2012; Paproki et al., 2012; Paulus et al., 2014). In particular, this refers to automatic high-throughput plant 3D imaging systems, which can continuously monitor changes of plant phenotypes without damage (Hartmann et al., 2011). Moreover, multifunctional software designed and performed in a 3D phenotypic platform can capture images through onboard systems containing visible, IR and NIR three optional probes, and process the continuous image to a big data of phenome, which include multiple dimension data such as temperature, moisture content, density, shape, color, and so on.

Many methods have been proposed for the identification of seed maturity, and almost all are performed after seed harvest (Li et al., 2015a, 2015b). There are rare methods

for quantitative determination of the seed maturity while the seed is still growing in capsule, pod, or cluster of the mother plant, which would be more meaningful for seed production. In this study, we demonstrated that the 3D phenotypic platform is a powerful image-analyzing instrument for assessing seed maturity by integrating traits of capsules while they are still growing in the field. However, the experiment was carried out in a greenhouse with a relatively stable environment, and its adaptability needs to be further confirmed in field and multiyear experiments. In addition, the 3D phenotypic platform is an expensive and large experiment platform for monitoring capsule development. A more portable and inexpensive instrument that provides comprehensive indices would be of great use, particularly one designed to make a sound in accordance with the range of specific trait parameters, which could be used to guide farming operations and to predict seed harvest time.

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Fig. 5. Parameters changes of capsule shape during seed development. EP, MCC, and MCR represent the external polygon, minimum circumscribed circle, and minimum circumscribed rectangle, respectively (means $\pm$ SD).

Table 1. Correlation between parameters of capsule phenotypic traits and maturity of tobacco seeds during capsule development. The seed germination potential of seeds represents the maturity of which and that is used to analyze the correlation with capsule traits.

	Length	Width	Perimeter	Area	EP area	EP perimeter	MCR perimeter	MCR area	Eccentricity ratio	Compactness	B	G	R	Water content
Width	0.913*													
Perimeter	0.823	0.949*												
Area	0.946*	0.992**	0.936*											
EP area	0.944*	0.991**	0.946*	0.999**										
EP perimeter	0.978**	0.974**	0.903*	0.993**	0.992**									
MCR perimeter	1.000**	0.913*	0.823	0.946*	0.944*	0.978**								
MCR area	0.980**	0.975**	0.899*	0.990**	0.988**	0.998**	0.980**							
Eccentricity ratio	-0.493	-0.796	-0.874	-0.738	-0.745	-0.654	-0.493	-0.649						
Compactness	0.587	0.580	0.296	0.571	0.543	0.588	0.587	0.604	-0.220					
B	-0.509	-0.752	-0.888	-0.694	-0.708	-0.625	-0.509	-0.631	0.926*	-0.042				
G	-0.232	-0.505	-0.693	-0.427	-0.444	-0.348	-0.232	-0.361	0.815	0.178	0.945*			
R	-0.533	-0.775	-0.913*	-0.725	-0.740	-0.656	-0.533	-0.657	0.945*	-0.042	0.994**	0.918*		
Water content	0.232	0.454	0.677	0.391	0.414	0.324	0.232	0.334	-0.739	-0.312	-0.918*	-0.981**	-0.893*	
Seed maturity	-0.843	-0.942*	-0.992**	-0.929*	-0.939*	-0.904*	-0.843	-0.906*	0.833	-0.295	0.888*	0.705	0.903*	-0.697

EP = external polygon; MCR = minimum circumscribed rectangle; B = blue; G = green; R = red.

*Significant at the 0.05 level (2-tailed); **significant at the 0.01 level.

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