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Multichannel imaging for monitoring chemical composition and germination capacity of cowpea (*Vigna unguiculata*) seeds during development and maturation



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ABSTRACT

This study aimed to set a computer-integrated multichannel spectral imaging system as a high-throughput phenotyping tool for the analysis of individual cowpea seeds harvested at different developmental stages. The changes in germination capacity and variations in moisture, protein and different sugars during twelve stages of seed development from 10 to 32 days after anthesis were non-destructively monitored. Multispectral data at 20 discrete wavelengths in the ultraviolet, visible and near infrared regions were extracted from individual seeds and then modelled using partial least squares regression and linear discriminant analysis (LDA) models. The developed multivariate models were accurate enough for monitoring all possible changes occurred in moisture, protein and sugar contents with coefficients of determination in prediction R_p^2 of 0.93, 0.80 and 0.78 and root mean square errors in prediction (RMSEP) of 6.045%, 2.236% and 0.890%, respectively. The accuracy of PLS models in predicting individual sugars such as verbascose and stachyose was reasonable with R_p^2 of 0.87 and 0.87 and RMSEP of 0.071% and 0.485%, respectively; but for the prediction of sucrose and raffinose the accuracy was relatively limited with R_p^2 of 0.24 and 0.66 and RMSEP of 0.567% and 0.045%, respectively. The developed LDA model was robust in classifying the seeds based on their germination capacity with overall correct classification of 96.33% and 95.67% in the training and validation datasets, respectively. With these levels of accuracy, the proposed multichannel spectral imaging system designed for single seeds could be an effective choice as a rapid screening and non-destructive technique for identifying the ideal harvesting time of cowpea seeds based on their chemical composition and germination capacity. Moreover, the development of chemical images of the major constituents along with classification images confirmed the usefulness of the proposed technique as a non-destructive tool for estimating the concentrations and spatial distributions of moisture, protein and sugars during different developmental stages of cowpea seeds.

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1. Introduction

As one of the most socio-economically important leguminous crops in Africa, cowpea (*Vigna unguiculata*) known also as black-eyed pea is a worldwide-distributed crop especially in the developing countries with multiple utilizations as a grain crop, vegetable crop and/or livestock fodder. The crop has numerous purposes as

a vegetable, snacks, and meal ingredients and the dry mature seeds are also suitable for boiling and canning as a ready-to-eat meal. Cowpea is affordable and considered as a sustainable source of essential phytonutrients, minerals and proteins, which are keys in solving malnutrition and food insecurity threatens [1–3]. Although such crop has a broad adaptation to various growing conditions, productivity of the cultivated fields with cowpea is still rather low, with an annual dry seed production of 7 Mt and an average yield of 5.68 t ha⁻¹ [4] mainly due to technological barriers or due to seeds with low physiological disorders or low quality.

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As the seed is the central input in agricultural production of all leguminous and field crops, great concern about seed quality has been raised from all stakeholders involved in crop production including breeders, farmers, variety registration agencies, and distributors because seed quality is the key factor for high crop productivity and increasing profit. As the seeds will face a plethora of unknown growing factors especially the adventitious agro-climatic conditions with the attendant risk of biotic and abiotic stresses, it is quite important to ensure high quality of such seeds before being cultivated to defeat such severe situations. Indeed, high quality seeds help in reducing production costs and guaranteeing more consistent and uniform crop stands, resulting in higher crop yields [5]. In this regard, seeds normally reach maximum quality at the end or shortly after the seed-filling period, a stage that has been termed as physiological maturity. Thus, seeds must reach the full physiological maturity before being harvested to assert a high germination rate, vigour, storage potential, field establishment and high yield in the subsequent growing seasons. Hence, obtaining seeds with such definite characteristics for a given genotype depends preliminary on defining the ideal harvest time at which the seeds accumulate the maximum dry mass and the highest nutrient reserves with high germination and vigor capacities [6] which is also influenced by the agro-climatic conditions. Immature seeds harvested earlier would have unbalanced chemical composition and are likely to be liable to mechanical injury permitting the entry of microorganisms. Whereas delaying harvest increases the risk of seed shattering or deterioration in the field. Even with a typical harvesting date of every genotype, seed-to-seed variation still exists in the same plant resulting in physiological differences among seeds with different nutritional value, germination capacity, viability, longevity and vigor [5,7]. In general, seed development during its life cycle is usually accompanied with a cascade of complex morphological, structural, metabolic and biochemical changes such as accumulation of different constituents such as protein and carbohydrate that should be accurately monitored [8]. Therefore, greater insight into intrinsic characteristics of the seeds before being sowed could help in improving crop yield and enhancing field performance [9].

Unfortunately, the current standard methods to assess the essential quality traits of the seeds are often dependent on time-consuming, destructive and cost ineffective techniques relying on either lab-based assays or subjective human inspection. With the ever increasing concern about seed quality, the demand for an advanced technique for non-destructive estimation of various seed properties is accordingly increasing. Spectral analysis applied not only for seed lots but also for single seeds allows ensuring overall quality and enables the assessment of seed uniformity with respect to a given property at earlier stages before being planted [10–12]. Several advantages such as reliability, non-destructiveness, precision, rapidity and avoidance of hazardous chemicals have favoured the use of spectral techniques in numerous applications in seed science and technology. Besides optical phenotyping, spectral techniques are able to provide essential information about the major constituents in seeds such as fat, protein, carbohydrates and moisture contents owing to their remarkable absorptions in the near-infrared region [13].

Due to the absence of spatial information in the traditional near infrared regions (NIR) spectroscopy, it was quite vital to develop a computer-integrated spectral imaging system combined with computational image processing and data analysis protocols to assess both external and hidden features of seeds [9,14–16]. Spectral imaging-based seed phenotyping techniques offer non-invasive solutions for large-scale quality screening to considerably reduce the cost, time, and labour. Deploying such time-efficient systems at different spectral ranges allows simultaneous determination of various seed traits [9]. Also, the non-destructive nature of

these methods helps in repeated sensing the seeds at different stages without causing any detrimental effects, which could not be realized with the traditional destructive techniques [17]. To overcome the huge size of the acquired hyperspectral images due to redundant information involved, multispectral imaging with few dedicated multi-channels has been emerged to tune computational challenges in image processing and data analyses. In “multispectral imaging”, the images could be acquired point by point, line by line or wavelength by wavelength (sequences of wavelengths). Thus, “multichannel imaging” represents one mode of image acquisition of ‘multispectral imaging’ in which the multispectral image is exclusively acquired wavelength by wavelength at very limited number of bands (channels). The multi-channel imaging system is tuned to specific “discrete” wavelengths over an extended spectral range from ultraviolet (UV) to NIR regions for the applications. This means that only few bands are employed to obtain the necessary information from the seeds being examined [18]. As a result of the availability of both spatial and spectral information across different bands in the spectrum [19] a substantial amount of research endeavours using multispectral imaging systems have been induced for characterizing different seed traits such as the prediction of seed viability and germination ability [20] detection of seed health, diseases and damages [21,22] and for visualizing structural details of specific compounds within a seed [19]. However, applications of spectral imaging for non-destructive estimation of different quality traits in cowpea seeds are very scarce [16].

Despite previous studies conducted for estimating the overall quality of matured seeds, few studies have been carried out for the determination of seed quality at different maturation stages, which is very critical for seed industry to ensure seed uniformity [15]. The dilemma is that the intra-plant variation in the field causes heterogeneous seed quality and seed depreciation due to agro-climatic conditions and production environment [23]. Therefore, the resulting seeds may vary in quality even with those ones harvested from the same plant in the same date [6]. To date, this issue has been investigated with traditional wet-chemical assays, but there are neither an available spectral method nor imaging-based techniques have been reported for a single-seed level [7]. Thus, this study was embarked to set a computer-integrated multi-channel spectral imaging system as a high-throughput method for the analysis of individual cowpea (*Vigna unguiculata*) seeds at different developmental stages. In view of the above-mentioned issue, spectral analysis and relevant multivariate calibration modelling will be carried out for the prediction of the quality of cowpea seeds in terms of germination capacity and unravelling the changes in essential constituents such as moisture, protein and sugars during seed growth and maturation.

2. Materials and methods

2.1. Materials, seeds and planting

Sixty-four plants of cowpea (*Vigna unguiculata*) cultivar Cream 7 provided by the Ministry of Agriculture and Land Reclamation, Egypt were grown in 20 cm diameter pots inside a glasshouse at PHENOTIC seed and plant phenotyping platform in the Research Institute of Horticulture and Seeds (Angers, France) following all recommended agronomic practices without applying pesticides or herbicides during the growing period. Plants were left *in situ* and the blooming flowers started to appear after 51 days of sowing were marked individually every day with tags bearing the date of flowering to identify the anthesis date. The developed pods containing 8–14 seeds were collected at different maturity times at 12 sampling dates every 2 days interval from 10 up to 32 days after

anthesis (DAA). Pods were taken to the seed testing laboratory, where seeds were immediately detached from the pods, imaged and analysed for growth and biochemical assays. Within an inflorescence, there was a seed-to-seed variation in maturation that may occur on pods harvested from the same plant. Because the quality evaluation of the seeds was planned to be carried out on individual seed basis, seeds were grouped to ensure that they belong to the same stage of seed development.

Directly after harvest, seeds were desiccated for 3 days at 35 °C and 45% relative humidity reaching an equilibrium moisture content of 12.5% to facilitate further handling and to avoid any influence of the varied moisture contents during image acquisition. Some seeds from each stage were kept fresh without desiccation to be used directly for the estimation of the exact moisture content of the seeds at every developmental stages. Generally, the seeds were divided into four parts. One part consisting of 600 seeds (50 seeds × 12 stages) was used for evaluating the germination capacity of cowpea seeds in terms of germination percentage and seed vigour. The second part consisting of a total of 180 seeds (15 seeds × 12 stages) was used for predicting moisture contents and dry matter. The third part consisting of 120 seeds (10 seeds × 12 stages) was used for the prediction of protein content and the last part consisting of 60 seeds (5 seeds × 12 stages) was used for predicting different individual sugars (i.e., sucrose, raffinose, verbascose, and stachyose).

2.2. Methods and data collection

2.2.1. Image acquisition

The multichannel imaging system used in this study for the acquisition of multispectral images of cowpea seeds at different developmental stages is an illumination-based spectral imaging system (VideometerLab3, Videometer A/S, Hørsholm, Denmark) from the PHENOTIC Platform employed in the UV, visible (VIS) and NIR regions. The system was supplied with a series of monochromatic light-emitting diodes (LEDs) at twenty wavelengths (375, 405, 435, 450, 470, 505, 525, 570, 590, 630, 645, 660, 700, 780, 850, 870, 890, 910, 940, and 970 nm) that were sequentially flashed one after another to record monochromatic images by a CCD camera at these pre-defined wavelengths. The resulting multichannel image is a cube image in HIPS format with a spatial dimension of 2056 × 2056 pixels, 0.0432 mm/pixel of spatial resolution and 20 bands in the spectral dimension. To acquire maximum quality images of cowpea seeds, the standard procedure for image acquisition split into an initialization step (to set up the imaging system) followed by the acquisition step itself. The initialization procedure involved calibrating the system radiometrically and geometrically to avoid optical misalignment and spatial distortion by using three reference plates (white, dark and dotted plates) in addition to adjusting lightening intensity at every operated LED to limit specular reflections and saturated pixels [24]. After performing the required spatial and spectral calibrations, the seeds assigned for specific determinations were arranged in a Petri dish with an adequate distance among them and one multispectral image was acquired for cowpea seeds at every developmental stages. At the end of the acquisitions, 12 multispectral images were obtained for the 12 developmental stages and saved for the subsequent analyses. For instance, Fig. 1 shows the processing pipelines involved in acquiring and analysing multispectral images for the prediction of moisture, protein and sugar contents in cowpea seeds harvested at different developmental stages.

2.2.2. Spectral data extraction

As the acquired multispectral image contains data at specific multi-wavelengths in the UV and NIR regions, it provides the required information for characterizing the major constituents in

cowpea seeds. Because the imaging system employed in this study was in the reflectance mode, the extracted spectral data could be also termed as spectral reflectance data. In addition to spectral data reside in every image cube, the image supplies spatial features of the target seeds from which some important morphological features such as colour, dimensions and texture can be easily extracted. As the seed consists of a complex structure and different regions with varied chemical composition, extracting a spectrum from only one location (pixel) is not a representative approach to represent the spectral fingerprint of the whole seed. Also, determining chemical analyses on the pixel level is practically impossible. Hence, it was important to extract spectral data from all pixels belonging to each individual seed and conducting the reference chemical measurements for the whole seed as well as illustrated in Fig. 1, the processing pipeline of multispectral images composed of several steps to extract spectral data and to build a meaningful multivariate calibration models for the assessment of the major quality attributes of cowpea seeds.

To extract spectral data from every individual seed in a multispectral image I_{ijk} , it was very critical to isolate seed objects from the background. Therefore, the image at wavelength of 780 nm (I_{ij780}) was segmented out by a simple thresholding producing white objects (seeds) with pixel values equal to 1 representing the seeds in a black background (non-seed pixels) having zero values. The segmented image was acted as a mask M_{ij} to define the locations of the seeds in the original multispectral image. The defined seeds by the mask were labeled and treated as a blob database from which one mean spectrum was calculated for every individual seed (blob) in the image as the average of spectra of all pixels belonging to every distinct seed (blob) in the image. By this way, spectral fingerprints of all pixels of a specific seed (blob) in the image had contributions on the average spectrum of this seed. Thus, the mean spectrum of a particular seed in the image was the mean reflectance intensity at discrete 20 bands from 375 nm to 970 nm that represents the spectral signature of this seed that reflects its physicochemical characteristics.

Based on the outlined method of spectral information extraction, four different spectral matrices (X_1 , X_2 , X_3 , and X_4) were formed with different utilizations of each. As shown in Fig. 2, three matrices (X_1 , X_2 , and X_3) were formed to collect spectral data that will be used for predicting chemical constituents (moisture, protein and sugars) in the individual seeds. The fourth matrix (X_4) was formed by collecting spectral data of all cowpea seeds assigned for the prediction of germination capacity. The first spectral matrix (X_1) was formed from spectra of 180 seeds (15 seeds × 12 developmental stages) for moisture determination. In the case of protein and sugar content determinations shown in Fig. 2, 120 spectra (X_2) were extracted from 120 seeds (10 seeds × 12 developmental stages) in addition to 60 spectra (X_3) were also extracted from 60 seeds (5 seeds × 12 developmental stages) in the same images. Spectral data in every spectral matrix were then split into training and validation datasets as explained in Table S1 and Fig. 2.

2.2.3. Measurements of seed chemical constituents

Fresh individual seeds were oven-dried at 90 °C for 48 h until achieving constant weight and the moisture contents of every single seed at different developmental stages were calculated as a percentage (wet basis) by using the difference between corresponding fresh and dried weights normalized to the fresh weight.

Seeds at different developmental stages were first dried in an oven at 90 °C for 48 h and samples of 1–5 mg of every individual seed were weighed in tin capsules to determine total carbon (C) and nitrogen (N) percentages in the sample based on the Dumas combustion method by an elemental analyzer (FLASH 2000

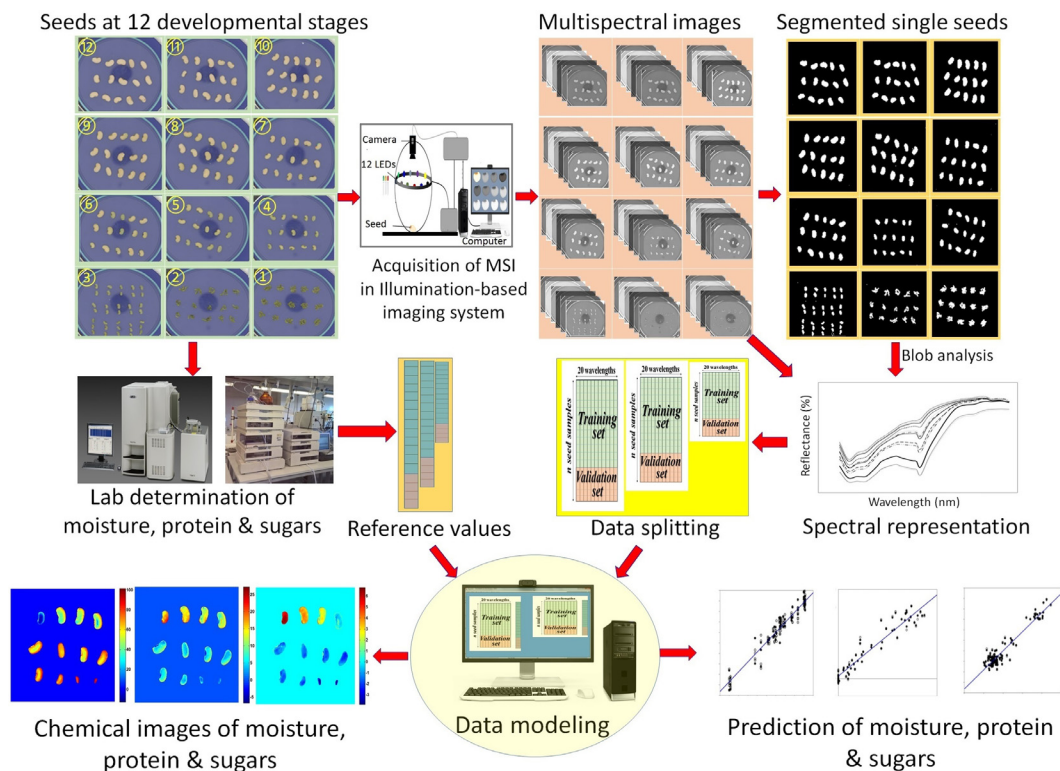


Fig. 1. Flowchart of the typical pipeline of all steps involved in processing multi-channel images and spectral extraction for analysing single seeds to predict moisture, protein and sugars in cowpea seeds during 12 developmental stages from 10 to 32 days after anthesis.

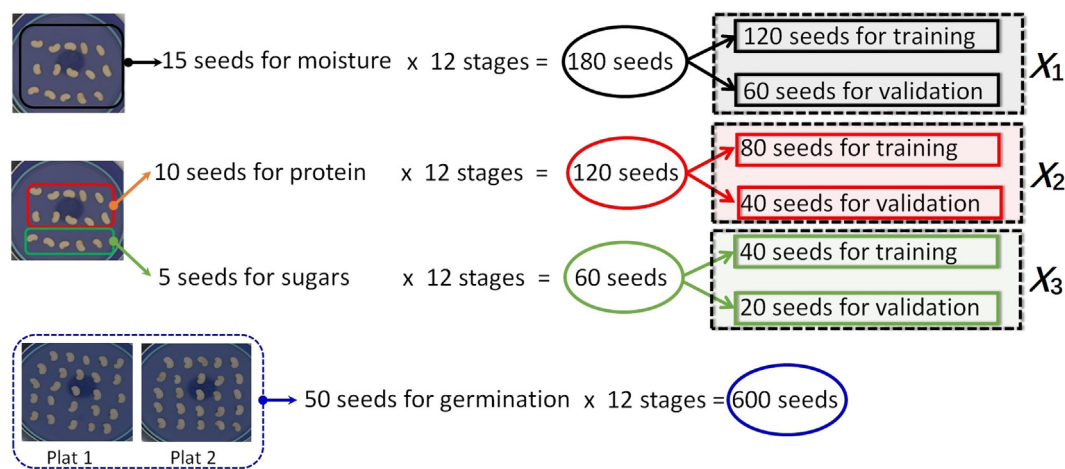


Fig. 2. Spectral matrices formed from spectral data extracted from multispectral images of cowpea seeds. Each spectral matrix assigned for the prediction of one constituent was split into training and validation datasets.

Organic Elemental Analyzer, Thermo Fisher Scientific, Courtaboeuf, France). The seed protein content was calculated by multiplying the nitrogen content by a conversion factor of 6.25 [7] and the result was expressed as a percentage of the original weight.

Individual seeds were split into triplicates, grounded and the powders were lyophilized. One millilitre of 80% methanol containing melzitose as the internal sugar standard was added to 20 mg of dried powder. After heating at 76 °C for 15 min, samples were evaporated under vacuum in a Speedvac AES1010 (Savant Instruments, Hyderabad, India). The residue was redissolved in 1 ml of distilled water, and after appropriate dilution, different sugars (i.e., sucrose, raffinose, verbascose, stachyose) were analysed by high-performance liquid chromatography (HPLC) on a Carbowac

PA-1 column (Dionex Corp., Sunnyvale, CA, USA) as described by [25].

2.2.4. Measurements of germination capacity

To determine the germination capacity in terms of germination status, germination percentage and vigour, two replicates of 25 seeds from each developmental stage were tested for germination in sterilized glass Petri dishes containing two moist filter papers each and incubated in a dark germination chamber at 20 °C. Seeds were evaluated individually based on the protrusion of the radicle and development to normal seedlings at the end of the test as markers of germination. Normal seedlings were characterized when all seedling structures such as cotyledons, primary leaves,

terminal bud, epicotyl, hypocotyl and roots were clearly visible and intact that can be developed to normal plants when grown under favourable conditions [26]. Also, seeds that were able to germinate early within three days were considered vigorous seeds. At every developmental stage, the number of normal seedlings were counted after nine days and used for calculating the final “germination percentage”. Accordingly, at the end of the test, every individual seed was classified as “germinated or non-germinated” and “vigorous or non-vigorous”. Moreover, the total number of germinated seeds and vigorous seeds at every developmental stage was used to calculate the overall germination percentage and the percentage of vigorous seeds at each stage, respectively.

2.2.5. Multivariate data analysis

Multivariate analysis is an essential step in all spectral data analysis to relate spectral information extracted from multispectral images of the examined seeds to the reference measurements of the chemical or physical attributes of interest [14]. There are different chemometric multivariate methods to mathematically decompose the complex spectral data into easier interpretable structures that can improve the understanding of chemical and biological information of the tested seeds [27,28]. Although, the acquired images are both voluminous and multidimensional, the availability of advanced computing systems with high-speed processors and enormous storage power, data volume is no longer a constraint. The problem of high dimensionality and complexity of the redundant information is not a severe problem in multispectral images as the number of bands used in image acquisition is quite small. In the current study, one multispectral image has a fixed dimension of 2056 pixel \times 2056 pixel \times 20 bands. The spectral data extracted from each individual cowpea seeds of different developmental stages were arranged in a matrix \mathbf{X} and their corresponding reference traits are arranged in a matrix \mathbf{Y} . Each column y_i of the matrix \mathbf{Y} holds the reference measurements of only one attribute (moisture, protein, sucrose, raffinose, verbascose, or stachyose). The reference values of one attribute y_i were separately modelled with the spectral matrix \mathbf{X} using partial least squares (PLS) regression under full cross-validation scenario using the leave-one-out method. Despite the developmental stage at which the seeds were harvested, seeds (n) assigned for each test were randomly divided into training ($2/3n$) and validation ($1/3n$) sets to help in building robust PLS calibration models. To ensure fair partitioning of the data, t -test was carried for each group to ensure that there was no significant difference between the two groups in all examined chemical constituents. In general, data were partitioned to develop PLS calibration models on the training group, and such developed models were then applied in predicting the chemical compositions of the seeds in the validation group.

The PLS regression has a mean advantage of combining features from principal component analysis (PCA) and linear regression. As collinearity is possible to exist among the 20 wavebands (predictors) involved in the test, PLS regression is suitable for this kind of data analysis to predict the dependent variable (the chemical composition seeds arranged in the vector \mathbf{Y}) from the predictors (spectral data arranged in \mathbf{X}). By decomposing spectral data (\mathbf{X}) and the reference chemical values (\mathbf{Y}), the PLS modelling extracts a new set of orthogonal variables called principle components or latent variables (LVs) that have the best predictive power and removes noises from both of these matrices. The greater number of LVs included in the model, the more complex the PLS model will be. Therefore, selecting the ideal number of latent variables in the model is very critical to minimize the expected error and to avoid under-fitting and overfitting of the prediction process. Using a large number of latent factors may provide good performance in fitting the current attribute, but it usually leads to overfitting because the model considers significant amount of noise rather

than the real spectral information. On the other hand, the under-fitting means the model does not have enough information for accurate prediction. That is why developing the PLS models under cross validation routine is a more statistically sound method for choosing the ideal number of latent variables. Moreover, the resulting PLS calibration model was then used to predict the constituent content in a new unknown seed sample using its spectrum at the 20 wavelengths.

As shown in Table S1, the number, range, mean and standard deviation (SD) of moisture, protein, and sugars in seed samples in the training and validation sets were tabulated. The number of seeds assigned in the calibration sets varied among tested parameters based on the availability of the seeds at the time of experiment. The reference values of seed chemicals had a broad range of variation, which was helpful to develop feasible and robust calibration models. The range of moisture, protein and sugar contents in the training set covered that of the validation set, and there were no significant differences between the two sets in regard to these parameters indicating that the distribution of the two sets was appropriate to develop reliable calibration models. In general, the accuracy of PLS models was assessed based on the values of determination coefficient in training set (R_c^2), in cross-validation framework (R_{cv}^2) and in the validation set (R_p^2). Cross-validation is a very vital tool to assess the optimal complexity of a model and identify the ideal number of principal components or LVs in the model [29]. The number LVs identified under cross validation as well as the root mean square error of calibration (RMSEC), root mean square error of cross validation (RMSECV) and root mean square error of prediction (RMSEP) were all used for evaluating every developed PLS models for the different attributes. Models with high performance were identified when they have higher coefficients of determination, minimum errors and least number of principle components. Image processing, blob analysis and extraction of spectral data from multispectral images were carried out using VideometerLab3 software (Videometer A/S, Hørsholm, Denmark) and the data analyses and model development was carried out using Unscrambler 7.9 software (CAMO, Trondheim, Norway). Chemical images were produced using a script developed in Matlab version 7.7.0. R2008b (The Mathworks Inc., Natick, MA, USA).

2.2.6. Statistical analysis

Analysis of variance (ANOVA) testes were carried out by using statistical analysis toolbox of Matlab (The Mathworks Inc., Natick, MA, USA) to investigate the difference among cowpea seeds harvested at 12 different developmental stages (10, 12, 14, 16, 18, 20, 22, 24, 26, 28, 30, and 32 DAA) in regard to every tested attributes. The data were analysed as a completely randomized design with 12 treatments (12 different developmental stages) of 15 replicates each (15 seeds) in case of moisture, 10 replicates (seeds) in case of protein, and 5 replicates (5 seeds) in case of sugars. The orthogonal differences among means were compared using least significant difference (LSD) method at $P \leq 0.05$.

3. Results and discussion

3.1. Changes in seed characteristics during development

Continuous observation during cowpea seed maturation through its development exhibited many morphological changes on pods and seeds from stage to stage in terms of size, colour and appearance. For instance, great variation in seed size was observed throughout developmental stages with a remarkable increase at later stages of maturation. Seeds of early stages were very small, light green and shiny, but at later stages seeds

increased in size, turned creamy with thin seed coat and developed a clear black eye. The increase in seed size was accompanied with a continuous increase in fresh and dry weight of the developing seeds with a linear decrease in seed moisture content from 90.953% at 10 DAA to 10.466% at 32 DAA (Fig. 3a, b), which is in accordance with data reported by [30]. As moisture content was

calculated as a percentage of fresh weight, there was an inverse relationship between the percentage of dry matter and moisture content and the decline in moisture content is ascribed to the fact that the rate of dry matter accumulation is higher than the rate of water accumulation in addition to water losses in later developmental stages due to dehydration. Practically, seed dehydration is

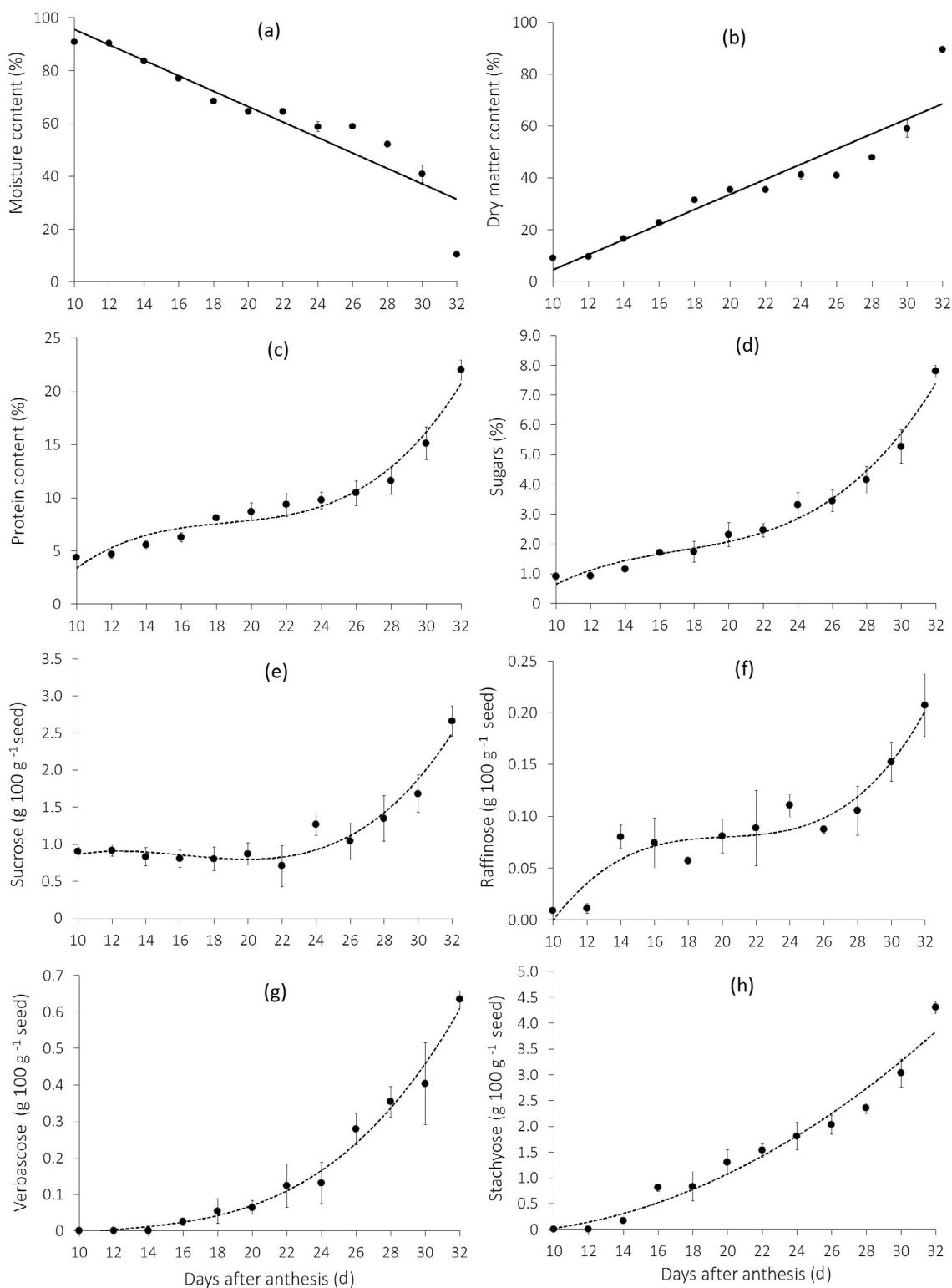


Fig. 3. Changes in (a) moisture content, (b) dry matter, (c) protein, (d) sum of sugars, (e) sucrose, (f) raffinose, (g) verbascose and (h) stachyose in cowpea seeds during twelve developmental stages from 10 to 32 days after anthesis.

the last event in cowpea seeds that marks the switch from a developmental mode to a germination one.

The measured contents of protein and sugars in seeds during development complied with the results reported in other studies [2]. With increasing seed maturity, there was a corresponding non-linear increase and accumulation of protein and sugars in seeds throughout the investigated developmental stages (Fig. 3c, d). The increase in storage protein content during development is ascribed to nitrogen accumulation in cowpea seeds denoting that final storage protein content can be adjusted by selecting the proper harvest time [31]. Similarly, total sugars per grain increased substantially after the onset of anthesis, reaching a maximum concentration at the end of the developmental stage. The changes in specific sugars (i.e., sucrose, raffinose, verbascose, stachyose) are shown in Fig. 3e–h in which it was clear that these sugars increased in a non-linear manner by increasing seed maturity. However, the rate of accumulating sucrose and raffinose was lower than that of verbascose and stachyose. The increase in verbascose was similar to the pattern of stachyose observed in the developing cowpea seeds (Fig. 3g, h).

Results obtained in this experiment also revealed that there were significant differences ($P < 0.05$) among seeds harvested at different developmental stages in terms of germination percentage and the percentage of vigorous seeds as shown in Fig. 4 and Table S2. Cowpea seeds harvested at 10 DAA until 16 DAA did not have the ability to germinate that might be attributed to the presence of some inhibitors such as abscisic acid [32] and the lack of sufficient nutrients and stored reserves to support embryo's continued development and post germination growth. The results revealed that as maturation proceeded, the germination of seeds was improved. Thus, the germination percentage increased rapidly reaching to 100% for seeds harvested from 26 DAA until 32 DAA. During this stage, the percentage of vigorous seeds was also very high reaching a maximum of 100% at 30 DAA and 32 DAA. This stage marks the end of physiological maturity because seeds achieve maximum viability and vigour [33]. Seeds with high germination and vigour generally provide early and uniform stands, signifying that the seeds will have the potential to produce vigorous seedlings. Based on the results obtained, it seems that seeds harvested at 30 DAA had the highest quality because these seeds had both high germination percentage as well as high percentage of vigorous seeds. However, as the moisture content of cowpea seeds at 30 DAA was 40.73%, harvesting at this high seed moisture content is very perilous and could lead to irreversible mechanical damage to seeds in addition to the liability of possible invasion of microorganisms and low storability. Moreover, average value

of protein content in the seeds at this stage was only $15.169\% \pm 1.56\%$. At 32 DAA, seeds had a perfect desiccation with moisture content of $10.466\% \pm 0.686\%$ and the protein content continued to increase up to $21.998\% \pm 0.932\%$. Besides, the germination percentage and percentage of vigorous seeds were both 100%. Thus, harvesting at 32 DAA is more preferable to produce high quality, nutritious and durable seeds compared to those ones harvested at 30 DAA. All biochemical changes as well as germination capacity of cowpea seeds during different developmental stages are explicitly shown in Table S2.

3.2. Description of seed spectral patterns

Fine details of spectral fingerprints of cowpea seeds at different developmental stages are very useful in predicting the maturation stage and provide information on the biochemical changes during development [34]. When seeds were sequentially illuminated by LEDs of different wavelengths (i.e., from 375 nm to 970 nm) during image acquisition, the light transmits through the seeds causing changes in the energy of the incident electromagnetic wave due to the stretching and bending vibrations of chemical functional groups containing bonds of O–H, N–H, and C–H related to different constituents in seeds. In illumination-based multispectral imaging, the intensity of individual pixels in the image depends basically on the absorption characteristics of the seeds being imaged besides the main characteristics of the illumination itself [18]. As the information available about how the maturation stage of cowpea seeds affects the absorption in the visible and near-infrared regions is rather limited, it is very crucial to highlight the matter in more details.

Every acquired multispectral image of a group of seeds consisted of a series of 20 grey-scale images at predefined wavelengths in the spectral range from the UV (375 nm) to the shortwave near infrared (970 nm). The average spectrum of a group of pixels belonging to one cowpea seed in a multispectral image is the spectral fingerprint of the whole seed that could be used to characterize its morphological, physical, and chemical characteristics. The average spectrum of one cowpea seed is illustrated as a plot between the magnitudes of reflectance versus twenty wavelengths. Spectra of cowpea seeds shown in Fig. 5 contain information from all chemical constituents of the seeds, and the direct interpretation of such spectra is quite useful to detect quality changes occurred in the seeds during maturation [7]. Accordingly, by analysing the reflectance patterns from every seed in the image, rapid and non-contact characterization of the seeds can be easily realized [35].

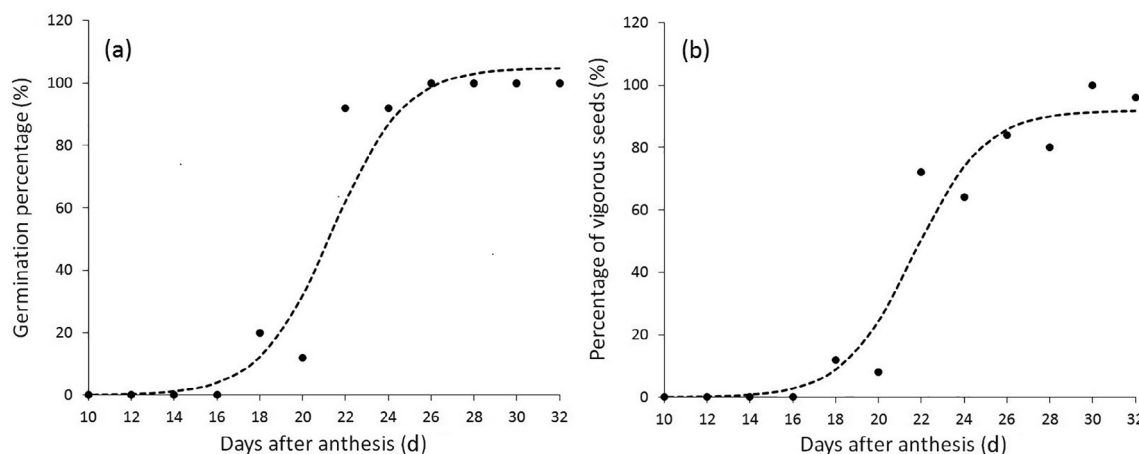


Fig. 4. Changes in (a) germination percentage and (b) percentage of vigorous seeds of cowpea seeds during twelve developmental stages from 10 to 32 days after anthesis.

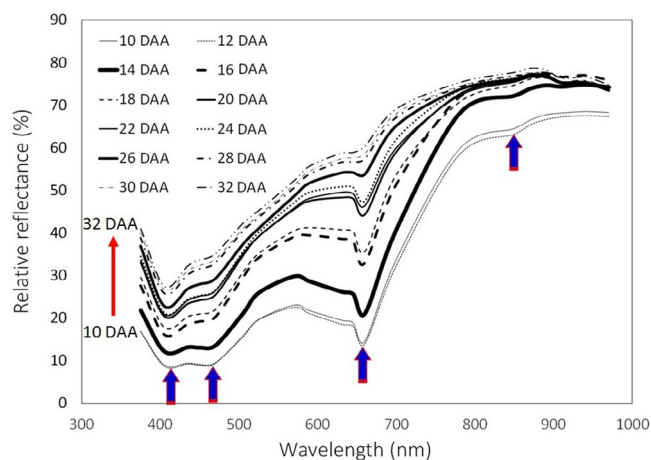


Fig. 5. Average spectral fingerprints of cowpea seeds harvested at different developmental stages from 10 to 32 DAA. DAA, days after anthesis. Blue arrows denote to the most significant absorption bands in the spectrum at 435, 470, 680, and 850 nm.

As shown in Fig. 5, reflectance spectra of seed samples showed very high variability throughout the whole wavelength range. This variability depends on the difference in physicochemical properties of the seeds from stage to stage. Significant absorbance regions were observed in the spectra where the changes of concentration of the main constituents could be followed [34]. These regions are due to presence of essential constituents such as pigments, water, protein, carbohydrates or even their interactions besides some other morphological and physical features such as colour, curvature and structure [11]. In general, spectral variations within the visible range (375–780 nm) are most likely attributed to the change occurred in the colour of the seed; whereas the variations in the NIR region (780–970 nm) were due to physicochemical changes [36]. As clearly seen in Fig. 5, the main absorptions of cowpea seeds at the twelve developmental stages were observed at approximately 435, 470, 680, and 850 nm in the average spectra. The absorption at 435 and 680 nm is related to Chlorophyll *a*, at 470 nm is ascribed to Chlorophyll *b* and at 850 nm is attributed to the presence of sugars. As cowpea seeds have very low fat and oil content [16] the absorption at 930 nm attributed to the presence of fat or oil (C–H) can't be discerned in all spectra despite the developmental stage. In general, as seed develops (e.g., from 10 DAA to 32 DAA), the magnitudes of reflectance increased (absorption decreased) at all wavelengths throughout the spectrum. In specific, the most noteworthy absorption bands at which the difference between seeds are obvious were those ones related to chlorophyll absorption at 435 nm and 680 nm.

3.3. Prediction of chemical constituents

The multi-channel spectral imaging method described in this study offers the opportunity to extract spectral fingerprints of all cowpea seeds at different developmental and maturity stages. The average spectrum of any seed in the image at any developmental stage reflects spectral patterns of a mix of pure constituents in the seed such as water, protein and carbohydrates including sugars (i.e., sucrose, raffinose, verbascose, and stachyose) at this stage. Thus, the chemometric PLS regression models developed to connect spectral fingerprints of cowpea seeds extracted at different developmental stages with their corresponding chemical measurements are extremely important in this non-destructive estimation of seed overall quality. To diagnose the ability of multi-channel imaging system and the accompanied multivariate analysis

method, a separate PLS regression model was developed for each constituent in the training samples first and then test this model in the validation samples. The results of predicting these constituents using PLS regression modelling are shown in Table 1 and the relationship between the measured and predicted values in the cross-validation and validation datasets are illustrated in Fig. 6. Statistical measures in terms of number of latent variables, coefficient of determination, root mean square error in calibration, cross validation and prediction presented in Table 1 revealed that the developed PLS models were accurate enough in predicting water, protein and sugars in cowpea seeds tested at different developmental stages with coefficient of determination in cross-validation of R_{cv}^2 of 0.94, 0.81 and 0.81, with RMSECV of 5.229%, 2.033% and 0.751%, respectively. When these models used in the independent validation set (seeds that have not been used during model development in the training step), the PLS models of water, protein and sugars had R_p^2 of 0.93, 0.80 and 0.78 and RMSEP of 6.045%, 2.236% and 0.890%, respectively. The minimum number of latent variables (5, 3 and 4), high values of coefficients of determination and slight values of error resulting from these models indicated that the developed models could be used safely in predicting such constituents during seed development with reasonable accuracy.

In addition to the prediction of sum of sugars in cowpea seeds, the concentrations of individual sugars such as sucrose, raffinose, verbascose and stachyose in every single seed were also predicted using separate PLS models. The results of PLS models in predicting verbascose and stachyose were good having coefficients of determination in prediction R_p^2 of 0.87 and 0.87 with RMSEP of 0.071% and 0.485%, respectively. However, the potential of using spectral data extracted from multispectral images for quantitative prediction of sucrose and raffinose was relatively limited and the accuracy of PLS models developed for these two sugars was not good enough like those ones of verbascose and stachyose in both training and validation sets with R_p^2 of 0.24 and 0.66 and RMSEP of 0.567% and 0.045%, respectively. This could be ascribed to the narrow range of sucrose and raffinose values observed in the seeds especially at earlier stages of seed maturation. The rate of accumulating sucrose and raffinose in cowpea seeds during maturation was lower than that of verbascose and stachyose. As shown in Fig. 3e, the values of sucrose in cowpea seeds at different maturity stages were almost equal during the period from 10 DAA to 26 DAA. Similarly, the values of raffinose did not exhibited great changes from 14 DAA to 26 DAA as shown in Fig. 3f. This attitude explains why the PLS prediction models of these two sugars were not robust enough. When statistical measures in the validation dataset are considerably lower than those of the training dataset, this may be an indication of model overfitting that should be alleviated if one decided to use it for further predictions [14]. In general, during different stages of maturation, the amount and composition of carbohydrates and sugars change in seeds due to the accumulation or depletion of different mono-, oligo- and polysaccharides [37] and following such changes during development is very useful in identifying the onset of obtaining seeds of high nutritional, physical and physiological features.

3.4. Classification of cowpea seeds based on germination

Cowpea seeds harvested at different developmental stages had different germination capacities where seeds harvested from 10 DAA until 16 DAA did not germinate, and seeds harvested at 30 DAA and 32 DAA had a full germination potential and provide vigorous seedlings as shown in Table S2. The classical method for the determination of germination capacity is traditionally performed

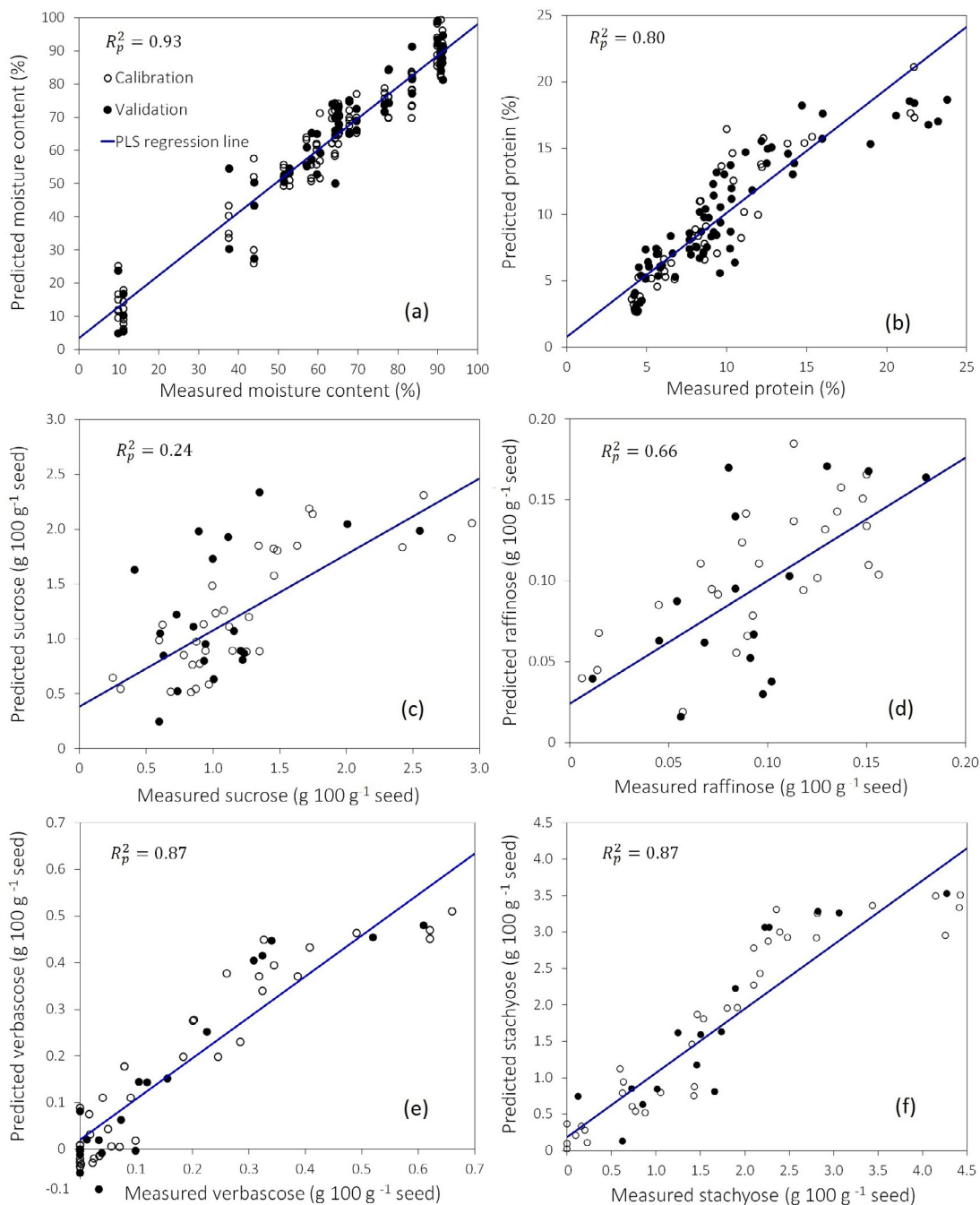


Fig. 6. Relationships between reference and predicted values from PLS regression models of (a) moisture, (b) protein, (c) sucrose, (d) raffinose, (e) verbascose and (f) stachyose cowpea seeds harvested at different developmental stages from 10 to 32 days after anthesis. Samples lying on the diagonal lines have equal measured and predicted values.

through laboratory assessment, which is an invasive and time-consuming process. Therefore, applying a rapid and non-destructive method to gather such information is critically important for seed industry. In essence, the variations in spectral features among seeds having different germination potentials are ascribed basically to the main difference in the physical and chemical properties of the seeds during the developmental stages. Thence, spectral data for individual seeds at the twelve developmental stages were analysed using LDA to classify seeds into only two classes: germinated (Class I) and non-germinated (Class II). This predefinition of the seeds was saved in a dummy variable (Y vector) and was modelled against seeds' spectral data (X). The total number of

seeds assigned for this test of 600 seeds (50×12 developmental stages) was divided into two subgroups: training set (400 seeds) and validation set (200 seeds). The LDA was developed in the training dataset under cross validation scenario and then the model was used to predict (classify) the status of the seeds in the validation set in terms of their ability of germination. During LDA analysis, the centroid of each class was first calculated, and a seed was assigned to a certain class when its average spectrum had the minimum squared distance to the centroid of this class [19]. The results of the LDA analysis are presented in the confusion matrix shown in Table S3. The tabulated results revealed clearly that the developed LDA model was very robust and had the capacity

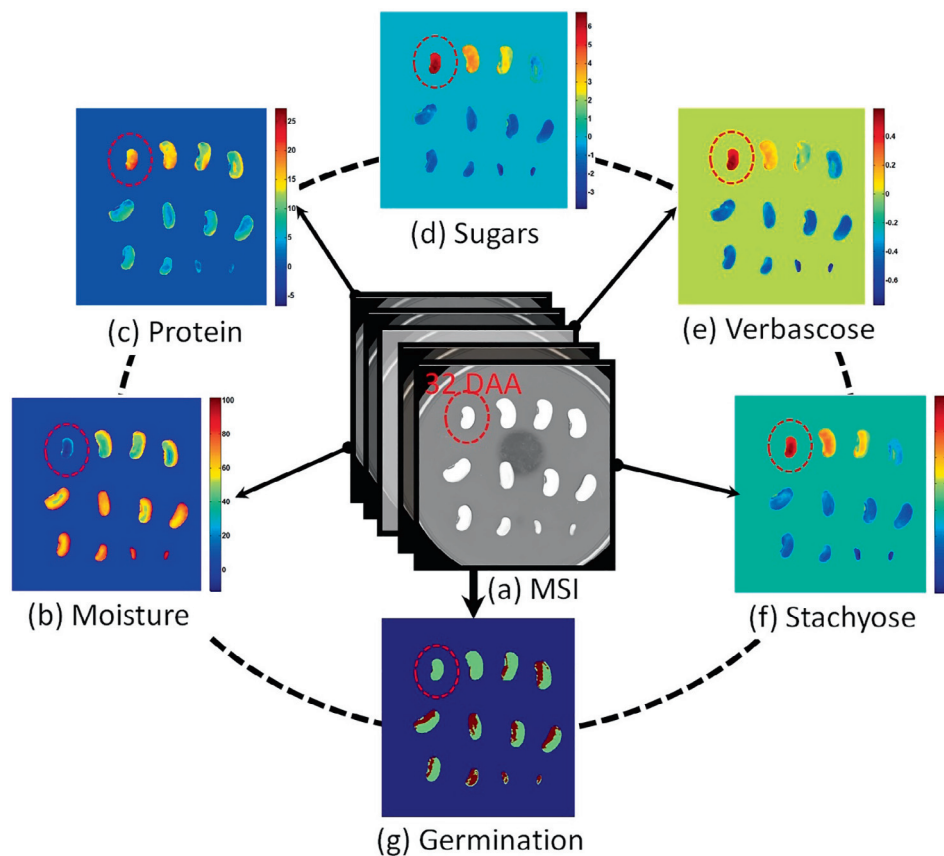


Fig. 7. Chemical and classification images produced from every multichannel image. (a) Raw multichannel image (MSI) of 20 grey-scale images at 20 different wavelengths containing twelve sequentially arranged cowpea seeds: one seed from each developmental stage. Application of partial least squares (PLS) regression models and linear discriminant analysis (LDA) model in every individual pixels in the original multichannel image resulted in chemical images showing the spatial concentrations of (b) moisture, (c) protein, (d) sum of sugars, (e) verbascose and (f) stachyose in addition to one classification image (g) showing the germinated and non-germinated seeds in the image. The red dotted circle encloses a cowpea seed at 32 days after anthesis.

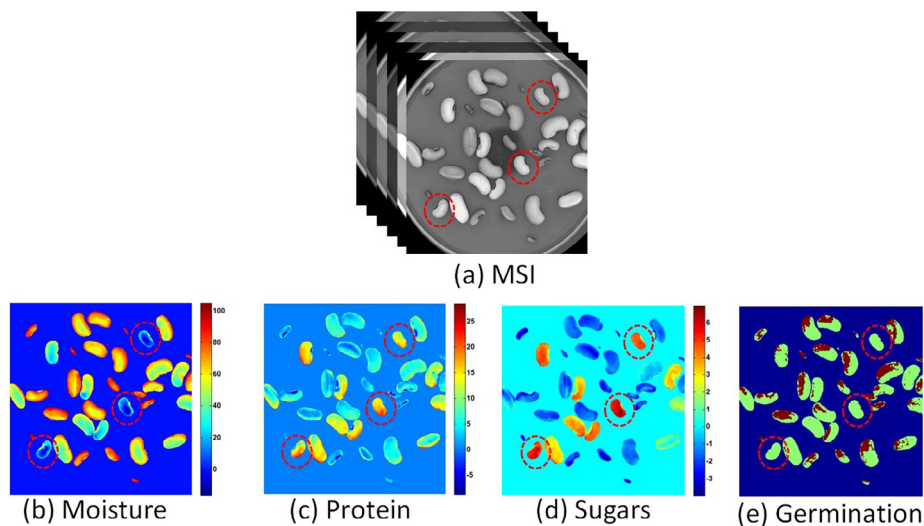


Fig. 8. Chemical and classification images produced from a multichannel image containing seeds of different harvesting stages. (a) Raw multichannel image containing a set of cowpea seed having different maturity distributed randomly in the scene. Application of partial least squares (PLS) regressions model and linear discriminant analysis (LDA) model in every individual pixels results in chemical images showing the spatial concentrations of (b) moisture, (c) protein and (d) sum of sugars in addition to one classification image (e) showing the germinated and non-germinated seeds in the image. The red dotted circles enclose cowpea seeds at 32 days after anthesis.

Table 1

Descriptive statistics of PLS regression models developed for predicting various chemical constituents in cowpea seeds at different developmental stages using 20 wavelengths in the UV, VIS and NIR ranges from 375 to 970 nm.

Constituent	LVs	Training		Cross-validation		Prediction	
		R_c^2	RMSEC	R_{cv}^2	RMSECV	R_p^2	RMSEP
Moisture	5	0.95	5.229	0.94	5.640	0.93	6.045
Protein	3	0.83	2.033	0.81	2.167	0.80	2.236
Sucrose	4	0.76	0.313	0.67	0.378	0.24	0.567
Raffinose	5	0.81	0.028	0.71	0.035	0.66	0.045
Verbascose	4	0.90	0.060	0.88	0.070	0.87	0.071
Stachyose	4	0.90	0.417	0.88	0.483	0.87	0.485
Sugars	4	0.85	0.751	0.81	0.884	0.78	0.890

PLS, partial least squares; UV, ultraviolet; VIS, visible and NIR, near infrared; LV, latent variables; RMSEC, root mean square error of calibration; RMSECV, root mean square error of cross validation; RMSEP, root mean square error of prediction; R_c^2 , coefficient of determination in calibration; R_{cv}^2 , coefficient of determination in cross validation; R_p^2 , coefficient of determination in prediction.

to correctly classify the seeds into the two pre-defined classes (germinated and non-germinated) in both training and validation datasets. The overall correct classification was 95.50%, 95.00% and 96.00% in the training, cross-validation and validation datasets, respectively. The similarity between the results in training and validation dataset undoubtedly indicates the robustness of the LDA model in detecting germinated and non-germinated seeds throughout all developmental stages. This demonstrated the potential of multispectral imaging methods outlined in this study for identifying the germination capacity of individual cowpea seeds before being sowed based only on their spectral signatures. Complementary with PLS models developed for predicting the major chemical constituents in cowpea seeds, the result of LDA reported here is crucial for the identification of the correct developmental stage at which the seeds had a high potential to germinate.

3.5. Prediction and classification images

The information resides in a multichannel image of cowpea seeds can be extracted in different forms and representations to express the intrinsic properties of the seeds such as colour, shape, dimensions, texture and spectral fingerprints. Conveying the developed multivariate models to every single pixel in the multichannel image will produce a new representation called chemical images, prediction images and classification images that would not have been exist without an efficient data modelling. The chemical images are pseudo-colour images in which each colour corresponds to a certain concentration of the mapped constituent. These new-emerged images incorporate both spatial and spectral information in a two-dimensional representation visualizing the spatial distributions and variability of all major constituents from point to point in the image which is otherwise impossible to achieve with the raw spectral data. As the multichannel images cannot be visualized at all channels at once, the prediction and classification images are forms of representation to show the contents of the image in a meaningful way that is simple to understand and easier to interpret for comparison and decision-making purposes. In the context of the current study, the main aim of developing prediction and classification images is to track the biochemical changes occurred in cowpea seeds throughout their developmental stages and how these changes are progressed from stage to stage to identify the best stage to obtain high seed quality.

Prediction images were mathematically created by multiplying the regression coefficients of PLS models developed during multivariate analysis step to every individual pixel in the multichannel image. As every pixel in the multichannel image contains spectral data at 20 wavelengths, the resultants of multiplication process give the concentrations of the constituents under interest in every

pixel in the image. Accordingly, with different PLS models (one model for each constituent) applied to the image, different prediction images (chemical images) could be produced showing spatial concentration distributions of different constituents for the same original image. In fact, obtaining the concentrations of major constituents in a pixel-scale resolution would not have been possible because it is theoretically impossible to obtain the reference values of constituents' concentrations in a very tiny portion (one pixel) of the seed using the ordinary wet-chemistry assessments. Similarly, when the classification model was applied to the image, the resulting image is called a 'classification image'. Consequently, applying the linear discriminant function resulting from the LDA model developed in this study for classifying the seeds according to their germination capacities results in producing classification image showing clearly the germinated and non-germinated seeds in the multichannel images. In this study, three prediction images (chemical images) were produced for moisture, protein and sum of sugars in addition to other four chemical images for four individual sugars (sucrose, raffinose, verbascose, and stachyose) showing their spatial concentrations in all seeds presented in the multichannel images during twelve developmental stages. Based on the statistical measures previously described, PLS models developed for sucrose and raffinose can merely be used for the prediction of these two individual sugars (Table 1), and they would give misleading results if applied in the pixel-wise level because it would produce deceptive chemical images. Therefore, five chemical images for moisture, protein, sum of sugars, verbascose and stachyose were produced from each multichannel image by applying their corresponding PLS regression models in all individual pixels in the image as shown in Fig. 7b–f. Besides, one classification image was also produced from the same image to classify the seeds presented in the image into germinated and non-germinated categories as shown in Fig. 7g.

Fig. 7a shows one raw multichannel image acquired at 20 different wavelengths containing twelve cowpea seeds: one seed from each developmental stage (10, 12, 14, 16, 18, 20, 22, 24, 26, 28, 30, and 32 DAA). The seeds were sequentially arranged from bottom to top with the bottom-right corner having the seed at 10 DAA and the upper-left corner contains the seed at 32 DAA. As shown in Fig. 7 b–f, applying five different PLS regression models in this multichannel image resulted in five different chemical images visualizing the variations in moisture, protein, sum of sugars, verbascose and stachyose in the twelve seeds presented in the image. In all chemical images presented in Fig. 7, the blue colour denotes low concentrations and red colour denotes high concentrations of the constituent. For instance, there were noticeable differences between the seeds in terms of their moisture contents (Fig. 7b) from a seed at 10 DAA having very high moisture content (>90%) and their pixels were visualized in red to a seed at 32 DAA

having a very low moisture content with their pixels visualized in blue. Similarly, with the aid of the colour scale attached to every chemical images, it was very easy from the first look to recognize cowpea seeds having different concentrations of protein and sugars from their corresponding chemical images shown in Fig. 7c and d, respectively. In addition to the chemical images, the classification image shown in Fig. 7g is very helpful in identifying the germinated seeds from the non-germinated ones. According to the visualized form shown in the classification image, the seed whose pixels were visualized in green was considered a germinated seed and those ones whose pixels were visualized in dark red were considered non-germinated seeds. The red dotted circle surrounding the cowpea seed at 32 DAA shown in the original multichannel image as well as all prediction and classification images indicate that seeds harvested at 32 DAA will have very low moisture content and high contents of protein, sugars and individual sugars. In addition, seeds expected to be harvested at 32 DAA will be able to germinate as all pixels of this seed appeared in green in the classification image shown in Fig. 7g.

To confirm the visualizations obtained from chemical and classification images and to avoid biased results due to predefined arrangement of the seed during image acquisition, a set of cowpea seeds having different maturities was distributed randomly in the scene and a new multichannel image of this group of seeds was acquired as shown in Fig. 8a. The regression and discrimination models were applied with the same manner to produce different chemical and classification images showing the spatial distribution of the major constituents as well as the categorization of the seeds to germinated and non-germinated seeds. Accordingly, three different cowpea seeds enclosed with a red dotted circle can be easily located in the image. These three seeds are characterized with lowest content of moisture (Fig. 8b), highest contents of protein (Fig. 8c) and sugars (Fig. 8d) and be classified as germinated seeds because all of their pixels appeared in green (Fig. 8e). This conclusion would not have been noticed directly for the original multichannel image without applying such sophisticated multivariate analyses. This result confirmed the robustness of the developed multivariate models in recognizing the identity of every individual seeds in the image despite their locations in the scene. In general, both forms of visualizations (i.e., chemical images and classification images) are extremely important for seed industry to take the proper actions if they decided to proceed in further investment in trade and agriculture.

4. Conclusions

The present study experimentally endeavoured multichannel spectral imaging system along with multivariate analyses using PLS regression modelling and LDA for accurate estimation of moisture, protein and sugars in cowpea seeds and classification of these seeds according to their germination capacity. Spectral fingerprints extracted from multichannel images provide insights into physico-chemical changes occurred in cowpea seeds during its development and maturation. Besides being very accurate in simultaneous prediction of various quality traits, the method outlined in this study demonstrates the potentiality of the proposed system in visualizing such traits in simple forms that will be very helpful for seed industry to take the proper actions if they decided to proceed in further investment in trade and cultivation of the examined seeds. Based on the results and findings of this study, this cost- and time-effective method is suitable to be employed not only for seed lots but also for single-seed scenarios. Thus, the state-of-art multichannel imaging method introduced in this study can be employed as a high-throughput phenotyping method to estimate the content and spatial distribution of different con-

stituents with strong promise to be adopted for seeds of other field crops for breeding trials, screening applications and production environments. Also, the applicability of this protocol to monitor germination and vigour in actual growing environments is critically important to identify vigorous seeds that can tolerate various biotic and abiotic stress under different environmental conditions.

CRedit authorship contribution statement

Gamal ElMasry: Methodology, Visualization, Writing - original draft. **Nasser Mandour:** Data curation, Investigation, Writing - review & editing. **Yahya Ejez:** Validation, Data curation, Visualization, Writing - review & editing. **Didier Demilly:** Validation, Formal analysis, Writing - review & editing. **Salim Al-Rejaie:** Conceptualization, Validation, Writing - review & editing. **Jerome Verdier:** Methodology, Resources, Writing - review & editing. **Etienne Belin:** Investigation, Funding acquisition, Formal analysis, Writing - review & editing. **David Rousseau:** Conceptualization, Supervision, Formal analysis, Project administration, Writing - review & editing.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data for this article can be found online at <https://doi.org/10.1016/j.cj.2021.04.010>.

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