Yielding to the image: how phenotyping reproductive growth can assist crop improvement and production

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Highlights

- High-throughput phenotyping of reproductive development has received little attention
- Phenology, pollen development, yield and components should be main targets
- Computer vision, machine learning and integration of sensing technologies have a positive impact
- Efforts in the analysis pipeline will facilitate adoption in crop breeding and production

Abstract

Reproductive organs are the main reason we grow and harvest most plant species as crops, yet they receive less attention from phenotyping due to their complexity and inaccessibility for analysis. This review highlights recent progress towards the quantitative high-throughput phenotyping of reproductive development, focusing on three impactful areas that are pivotal for plant breeding and crop production. First, we look at phenotyping phenology, summarizing the indirect and direct approaches that are available. This is essential for analysis of genotype by environment, and to enable effective management interpretation and agronomy and physiological interventions. Second, we look at pollen development and production, in addition to anther characteristics, these are critical points of vulnerability for yield loss when stress occurs before and during flowering, and are of particular interest for hybrid technology development. Third, we elaborate on phenotyping yield components, indirectly or directly during the season, with a numerical or growth related approach and post-harvest processing. Finally, we summarise the opportunities and challenges ahead for phenotyping reproductive growth and their feasibility and impact, with emphasis on plant breeding applications and targeted yield increases.

Keywords (6)

High-throughput phenotyping, reproductive structures, phenology, pollen, floret fertility, yield components

Contents

1. Introduction 3
2. Phenology 4
2.1 Internal stages 5
2.2 External stages: Inflorescence emergence and flowering 5
2.3 Physiological maturity 8
3. Pollen and floret fertility 8
3.1 Pollen production and viability 8
3.2 Anther extrusion, dehiscence and colour 10
3.3 Pollen shedding external to the inflorescence/flower 11
4. Yield and components 12
4.1 Prediction of yield via phenotyping canopy characteristics 13
4.2 Phenotyping yield components and underlying traits 14
5. Feasibility, impact and conclusions 21

1. Introduction

Reproductive organs are the main reason we grow and harvest most plant species as crops. Grains of rice, wheat and maize provide ca. 50% of food calories and account for ca. 60% of the annual harvested crop area; together with soybean, they further support calorie intake indirectly as feedstock for animal production [1 and references therein]. The steady decline in world relative grain yield improvement in wheat, rice and maize [1], compounded by climate change, calls for all disciplines supporting plant growth to make a contribution towards lessening the impact on future food security. Proximal and remote sensing have been contributing to different aspects of crop status diagnostics and production since the ‘70s (e.g. [2]). Phenotyping canopy features in the visible and near infrared (NIR) regions of the light spectrum has helped derive vegetation indices based on chlorophyll or nitrogen content which together with canopy temperature as indicator of transpiration are helping plant breeding programs improve the odds of selecting the right genotypes [3-5] and facilitate agronomy interventions [6] such as tactical irrigation [7] or fertiliser applications [8, 9]. In comparison, we lag behind in our efforts to remotely or proximally sense differences in the phenotypes of reproductive structures, their metabolic status, activity and even morphology. Many of these areas currently rely heavily on manual phenotyping, mainly by visual assessment. This is not only time and labour consuming, but also susceptible to error, variability and generally not high throughput. Advancements in phenotyping reproductive features would benefit those looking for underlying

genotypic differences for research purposes, mining genetic resources or in breeding programs satisfying a technological need such as hybrid production, but also when the response to the environment needs to be characterised for agronomy applications.

In this paper, we will use the physiological framework in which reproductive organs develop, grow and yield to summarise existing and upcoming methods to phenotype reproductive growth, preferably from medium to high throughput. In this framework, the yield of annual crops is mainly determined by grain number [10, 11], which is specifically vulnerable to environmental constraints in particular developmental windows. A summary of existing knowledge on this topic [12], confirmed by field data [13], showed that while in the cereals (e.g. wheat and barley) the period of greater vulnerability coincides with that of rapid spike growth, from early stem elongation to ca. 1 week after flowering; in pulses and legumes this period is displaced to later development stages, related to indeterminate habit and the ability to compensate pod number with seeds per pod during grain filling. Similar studies have been conducted in summer crops, confirming the dependence of grain number on plant growth rate during the critical period around flowering [14]. Based on this, we have divided the paper in four areas that cover most of the current critical subjects around crop reproduction. First, we review phenotyping phenology and associated processes, as pivotal for genotype by environment (GxE) and genotype by environment by management (GxExM) interpretation, and agronomy and physiological interventions. Second, we look at pollen development and production as a known critical point when stress occurs before and during flowering [15], which is of particular interest for hybrid technology development. Third, we elaborate on phenotyping yield components, their dynamics and post-harvest processing. Fourth, we summarise opportunities and challenges ahead for phenotyping reproductive growth and their feasibility. Where possible, methods are put together by scale, e.g. microscopic, plant or canopy level and both direct phenotyping and indirect diagnostics by association with other traits are mentioned.

2. Phenology

Crop developmental stages frame resource capture and the development of reproductive structures, therefore each stage constitutes a milestone in the formation of yield components. All crops have a vegetative, reproductive and grain filling phase; while in determinate cereals there is a clear distinction between these phases, overlap occurs in indeterminate pulses and oilseeds. Phenotyping

phenological stages is faced with many challenges e.g. the location of reproductive organs and variation of timing and duration of reproductive stages. In monocots with a terminal inflorescence there is a neat transition from vegetative to reproductive in the main axis (wheat, maize, etc.), whereas in many dicots, such as soybean and other oilseeds and legumes, the reproductive stage can occur throughout axillary nodes while the main axis is still producing leaves. Further to that, important dichotomies are visible vs. internal developmental stages, individual culm/plant vs. canopy, presence or absence of colour, visual contrast between the reproductive structure and the canopy, etc. Examples of the current status and areas of opportunity in remote sensing are highlighted in this section, especially those where major advances are critically needed.

2.1 Internal stages

In most crops, the transition from vegetative to reproductive and other steps in reproductive development are not exposed events, occurring while the apex is within the whorl or enclosed by leaves. Examples are early flower bud development in canola and double ridge or terminal spikelet in the inflorescence of determinate monocots such as wheat. Implementing non-destructive high throughput phenotyping methods targeting covered organs is not a trivial task. Results from X-ray micro-computed tomography in cut samples of barley spikes covered by sheaths are promising in terms of separating organs [16] but have not yet been targeted at younger stages and adverse effects on plants have been reported [17]. It would be desirable for this method to be applied to enable the non-destructive estimate of spike growth rates and derivation of partitioning coefficients, even if only under controlled environment conditions, but although low-resolution scans can be conducted rapidly, the ability to apply this for high throughput screening is at present questionable. At the apex level, traditional apex microscopy is currently benefitting from directed image analysis and modelling to explore specific morphometric features in a large number of genotypes [18].

2.2 External stages: Inflorescence emergence and flowering

Recording flowering is a task regularly undertaken in breeding programs, for agricultural management decisions and research trials. Flowering is primarily scored by eye following a crop specific scale, e.g. Zadoks in wheat and barley [19], and can be a highly labour consuming activity. The diurnal course of flowering has also become important in certain crops, in relation to seeking genotypic variation for stress avoidance mechanisms [20] and could be used to further understand seasonal performance

and interpretation of GxExM. Current available methods are summarised below ranging from utilisation of satellite images to more detailed information from analysis of close-up digital images.

Links between multi-spectral satellite-based imagery and crop phenology at paddock or regional scale have been made following time series of different vegetation indices, similarly to monitoring natural vegetation seasonality [see examples in Table 1 in 21]. A two-step filtering method to predict maize and soybean phenology modelling the calendar time progression of the Wide Dynamic Range Vegetation Index (WDRI) derived from 250-m Moderate-resolution Imaging Spectroradiometer (MODIS) images, resulted in a root mean square error from 3 to 7 days in maize silking and dent stage respectively [22]. Replacing the calendar time after sowing for a calculated thermal (maize) or photothermal (soybean) time for shape fitting WDRVI and testing it over a longer time series and more sites, increased the accuracy of phenology estimation to 2-4 days in maize depending on stage and location [23]. A similar approach based on Spectral Shape Indices, also based on MODIS bands, succeeded at detecting first square and first bloom stages in cotton [24]. Attempts have also been made to determine phenology by proximal sensing using hyperspectral point sensors or imagery. Visible atmospherically resistant indices (VARI), e.g. only relying on visible wavelengths, were more sensitive than the Normalised Difference Vegetation Index (NDVI) to detect tasselling in maize; tassels had higher reflectance at all wavelengths, significantly reducing the absorption of radiation in the red region [25]. VARI was sensitive to the fraction of flowers of oilseed rape in a given image based on images from a camera with green, red, red edge and NIR bands mounted in an unmanned aerial vehicle (UAV) [26]. Reflectance at 550nm was significantly better at predicting the flower fraction at both high leaf area cover and when there was exposed soil background. In wheat, the normalized heading index (NHI) based on the 1.2μm water absorption feature, had variable success (53-83%) at discriminating spike emergence (heading) in a semi-arid region, similarly when using ground based equipment or satellite imagery. While analysis of the evolution of indices to determine phenological stages can get quite detailed, e.g. that of red-edge Chlorophyll Index to determine active tillering, jointing and maturity in rice [21], it is probably more suited for ex-post interpretation of crop performance than real time decisions of crop management. However, attempts to implement short term [27] or closer to real time predictions [28] combining remote sensing data and innovative predictive techniques have been proposed.

Visually determined phenological stages are susceptible to human error but amenable to imaging technologies in various settings, from glasshouse to field. Images have been used to recognise and count green peppers embedded in a dense glasshouse canopy [29] and for estimation of oat panicle development in a plant phenotyping facility, using local texture patterns, with prospects for further development [30]. Amongst the challenges for image analysis are changing illumination throughout the day, canopy structure, level of exertion of the inflorescence and contrast of colour and texture between the inflorescence and the rest of plant or canopy, etc. Computer vision is a field emerging as an important contributor to this area, particularly using the train-then-classify method. Using maize as an example, three identified challenges for computer vision are (i) disambiguation, i.e. individuating plants from a mass, (ii) assignment, i.e. assigning an organ to a particular plant and (iii) identification, i.e. detecting and classifying different phenotypes [31]. In crops that grow in narrow rows at high density (e.g. wheat), detection of individual plants is neither practical nor possible. Moreover separating reproductive organs from the background of other organs (stems, leaves) and detecting superposition has clearly been a challenge that is only recently starting to be successfully addressed, as explained below.

In the field, it has been possible to automatically detect rice flowering panicles in a time-series of RGB (Red-Green-Blue) images captured every five minutes by a camera set effectively at 1m or less above the inflorescences [32], quantifying for the first time the number of panicles flowering each day and the diurnal trends on flowering with reasonable confidence. The method is based on training and testing populations of images and involves image acquisition, feature extraction and classification underpinned by machine learning. In wheat, heading stage from RGB images was captured with fixed cameras at 5m height, using a coarse to fine approach to detect ears, and equated the likelihood of 50% of the population of plants reaching the stage to that of detecting heading in 50% of the images [33]. Fixed camera systems have the advantage of capturing the dynamics of the same area and, depending on the height, increased resolution. Adapted to larger field experiments using a movable camera system, the method was expanded to include not only spike emergence (heading) but also spikes with extruded anthers [34]. Interestingly, the method can be applied to cultivars of contrasting spike and canopy characteristics. All of these methods have the potential to give a decimal code score at the plot level, i.e. not based on a single spike basis but as a proportion (generally 50%) of the spikes reaching a particular stage, i.e. to discriminate between different percentages of anther

extrusion [34]. Similar computer vision principles could be applied to detect early inflorescence stages in other crops, such as green bud in canola once the inflorescence is no longer covered by leaves. Currently, none of the proponents of phenology detection by computer vision have validated their methods using an unmanned aerial vehicle (UAV) setting, which would be most useful to cover a large number (100s-1000s) of small plots (1-12m^2) in different locations, such as those in plant breeding operations or agronomy trials. Finally, depending on the crop, other properties of the inflorescence could be used to increase the precision of detection of the inflorescence in early stages of emergence. For instance, in wheat and barley, spikes have different temperature than leaves based on stomatal density and other features [35], coupling thermal together with regular cameras could help to more precisely detect early stages of spike emergence or facilitate picking up spikes in lower layers of the canopy.

2.3 Physiological maturity

Seed physiological maturity is another developmental stage relevant for research and agronomic decisions around harvesting and sales, where the visual phenotypes is effective in some crops and not others. Indirect characterisation of duration of grain filling has been attempted under heat and drought by capturing not the status of the inflorescence but canopy senescence. A model based on spectral bands ranging from 550 to 1140nm at seed filling stage (R5-R6) predicted 50% of the variability in the days to maturity among soybean cultivars [36]. NDVI decay after heading has been used to predict days to maturity with varying success in three populations [37]. Methods to characterise the completion of grain filling based on the status of the grains still depend on destructive sampling to characterise the moisture percentage of the sample by weight. The methods commonly used for detection of harvest moisture content, electric capacitance or resistance and NIR, are valid once the inflorescence can be threshed, ca. 13-15% depending on the crop, i.e. at a later stage than the thresholds of moisture for physiological maturity, i.e. ca.40% in wheat [38].

3. Pollen and floret fertility

3.1 Pollen production and viability

With the renewed interest in hybrid cereal breeding, industry and academia are making efforts to better understand floral biology, in particular pollen production and viability. The ability for effective pollination is key for crop productivity and in the case of breeding and seed production this will in

many cases involve outcrossing. Ensuring good seed set requires maximising reproduction potential. This relies upon the number of pollen grains produced per anther and their viability, as well as their capacity to reach the appropriate stigmatic surface and pollinate. These traits are also of direct relevance to abiotic stress tolerance, as greater pollen production can mitigate pollen loss due to cold/heat induced sterility [39]. However, assessing traits such as anther size, amounts and viability of pollen are extremely challenging, particularly in a high-throughput field screen.

A number of approaches have recently been used to assess pollen number. Subsequent to extraction of pollen grains into an aqueous media such as potassium iodide, researchers have employed one of three methods i) manually counting extracted pollen, ii) automated counting using image analysis software and iii) automated counting using impedance flow cytometry. Manual counts using aliquots of pollen suspensions, have been demonstrated as sufficiently accurate to detect genotypic differences for wheat-rye addition lines [40] and wild grass species [41]. Nevertheless, the simultaneous screening of a large number of genotypes within a short time frame, as would be required within a breeding programme, may necessitate the application of automated counting. For thistles, Costa and Yang [42] were successful in developing an analysis pipeline to discriminate and subsequently quantify the number of grains within aliquots of pollen suspension imaged under a microscope, using the freely available ImageJ software (https://imagej.net). Image analysis was capable of quantifying the number of pollen grains within 60 sec or less per image at a reasonable level of accuracy and consistency, as compared to 5-68 min for unaided counting.

In addition to the number of pollen grains, the proportion of viable grains produced is of direct relevance to stress tolerance and outcrossing potential. Researchers typically employ chemical assays towards assessing this trait. While hand pollinations can provide a direct estimate of fertilization capacity, they require a much greater investment in both time and labour making the simultaneous screening of multiple genotypes impractical. Further to this, factors apart from pollen quality such as emasculation and fertilization technique, temperature and relative humidity can dramatically affect results.

The fluorescein diacetate test (FDA), or FDA combined with propidium iodide have been described as providing a reliable estimate of fertilization capacity [43] as it simultaneously tests for an intact pollen cell membrane and esterase activity, only fertile grains fluorescing during microscope inspection.

Conversely, assays such as Alexander's stain [44] or potassium iodide, staining for starch accumulation, are more suitable for assessments of sterility as opposed to viability, as they are only capable of distinguishing mature from immature pollen grains. Whilst the FDA test has been demonstrated to be correlated with fertilization capacity, as assessed by hand pollinations [45]; it is more suitable for hypothesis testing than breeding objectives or field trials as it requires a significant investment in time and access to fluorescence microscopy.

The impedance flow cytometry (IFC) technology provided by Amphasys (www.amphasys.com) may provide a more practical approach towards screening a large number of lines for pollen viability. Subsequent to extraction of pollen into a proprietary buffer, which preserves their integrity and filtration to remove cellular debris; single grains are passed through a chip to which radio frequencies are applied. Pollen viability is inferred from the behaviour of pollen grains while passing through this chip; for cucumber, tomato and sweet peppers strong correlation has been found between the results of IFC and that from FDA staining [46]. IFC also offers an alternative for the automated counting of pollen grains, promising a higher level of accuracy as each pollen grain within a sample is counted [46]. Presently, IFC may not be widely adopted, as it requires a substantial investment in the relevant technologies. While the development/optimization of image analysis pipelines can provide a more cost-effective and user-friendly approach towards assessing the number of pollen grains per anther, IFC still has the greatest potential for high throughput assessment of pollen viability, which can be conducted in a field environment.

The ultimate test of whether pollen is viable is pollen germination and fertilisation. Manual assessments of these are feasible by *in vitro* pollen germination tests, or manual cross-pollinations of sterile lines, nevertheless they are highly time consuming. There is also the issue of the duration of pollen viability and how rapidly this declines on and off the plant, which is currently difficult to assess using the available techniques. These are areas that would benefit from research to establish high-throughput phenotyping methods.

3.2 Anther extrusion, dehiscence and colour

A number of recent studies in wheat have assessed anther extrusion capacity within large genotype panels using a relatively simple approach [47-49]. Wheat ears are harvested subsequent to the completion of anthesis and the number of anthers retained within a pre-determined number of central

florets is counted; from this, the number of extruded anthers is determined. This approach has been demonstrated to be sufficiently accurate to detect significant genotypic differences and high trait heritability is typically observed with its application, higher than that observed with visual anther extrusion scoring [47-49]. Further, the approach is not exhaustively time consuming; requiring only 20 min to carry out counts on five ears, also assessments do not need to be carried out within a fixed period of time as ears could either be frozen or dried at room temperature [48].

Visually scoring the colour of wheat anthers has also been proposed [48]; hypothesizing that extruded anthers, having a dark yellow colour, may not have shed a large proportion of their pollen grains within the floret. Low trait heritability, the narrow time window in which scoring must be carried out and the large influence of environmental conditions suggest that alternative phenotyping approaches would be required. An alternative to scoring anther colour, would be to score anther dehiscence from which inferences on outcrossing potential can be made together with anther extrusion capacity. In rice, genotypic variation has previously been reported for the length of dehiscence in the basal and apical parts of the anther thecae [39] with high trait heritability being observed in each instance. Further to this, significant genotypic variation and GxE has been observed for the proportion of dehisced anthers [50]. The assessment of dehiscence length and the proportion of dehisced anthers appears feasible for a large number of genotypes within field trials; the only required piece of technology was a stereomicroscope in both instances and while sampling must to be conducted within a narrow time window, anthers can be stored until such time that they can be assessed. The improvements to phenotyping capacity that can be achieved by the development of image analysis pipelines targeting these traits, such as those successfully applied towards quantifying pollen grains, would be beneficial towards furthering our understanding of the extent of genotypic variation and the feasibility of selecting for these traits.

3.3 Pollen shedding external to the inflorescence/flower

In the early stages of the breeding cycles of predominantly self-pollinating crops, it is more practical to simultaneously select for traits known to determine pollen shedding capacity; in effect aiming to develop a male ideotype. Ultimately, in the later stages of breeding programmes, assessment of whether traits have been successfully integrated must be carried out. Within the literature two rudimentary but accurate approaches have been described; i) the use of adhesive surface based pollen traps [51, 52] and ii) measuring pollen mass at the end of anthesis [48].

Adhesive surfaces suspended within the canopy during anthesis provide not only an estimate of the number of pollen grains shed external to the flower structure but also insight into the temporal variation in pollen shedding. Prevailing environmental conditions inevitably influence the effectiveness of this approach, specifically wind speed and wind direction [52] as well as rainfall. Provided that conducive weather conditions occur during the study, or in instances where genotypic variation in flowering time is minimal, a high degree of resolution can still be achieved [51].

In an attempt to circumvent this inherent limitation of using adhesive surfaces, the pollen shedding capacity of wheat varieties was assessed through measurements of pollen mass [48]. This was achieved by placing wheat ears into paper bags prior to anthesis and subsequently measuring the mass of pollen collected at the bottom of the bags after the completion of anthesis. While the method does not provide information on the temporal variation in pollen shedding, it was able to detect significant genotypic differences and gave a modest trait heritability (0.76) supporting its accuracy. With the advent of a large number of crop imaging platforms designed for field trials, avenues for developing higher throughput and more accurate approaches towards assessing temporal and genotypic variation in pollen shedding should be explored. This would be of immediate benefit to the plant breeding community, in that it would be anticipated to considerably improve the capacity of breeders to phenotype for the trait that ultimately determines the efficiency of hybrid breeding schemes.

4. Yield and components

Genetic gains in yields from staple crops have been successfully achieved in past decades based on one empirical selection criteria: yield *per se.* Even though large-scale field evaluation of thousands of breeding lines are expensive in terms of time and financial resources, the use of combine harvesters is to date the most efficient way to evaluate yield from any seed crop at the end of the cycle. However, since yield is characterized by high GxE interaction and low heritability, empirical selection for *yield per se* will be insufficient to achieve much needed genetic gain, therefore traits linked to yield possessing higher heritability have an important role to play [53, 54]. Phenotyping more heritable traits can increase prediction accuracy in genomic selection strategies [55, 56] and has been successful in the detection of donors for strategic crosses schemes [57]. In addition, yield cannot be reliably assessed in single or paired rows in early generations, in contrast with some of the underlying

traits. To help reduce time and resources, high-throughput and inexpensive selection tools are needed to allow breeders to reliably screen large numbers of genotypes in a relatively short time in small plots. In this section we discuss progress in phenotyping (i) yield per se, (ii) yield components and traits underlying yield.

4.1 Prediction of yield via phenotyping canopy characteristics

Canopy properties, e.g. size and potential for photosynthesis and transpiration determine much of the processes culminating in yield. As such, diverse forms of characterising the canopy have resulted in correlations with yield. For instance, visible images have been widely used in plant science for its low cost and its ease of operation and maintenance, providing rapid measurements for plant phenotyping applications. RGB digital images at the canopy level and derived ground cover or vegetation indices were significantly correlated with yield in wheat [58] and maize under different N regimes [59].

Spectral reflectance profiles or derived indices associated with specific crop traits have been proposed as a fast and non-destructive technique that can be efficiently used in breeding programs where thousands of individuals must be screened every year [60]. Spectral vegetation indices, where different bands are combined in the red and NIR region of the spectrum, related to pigment absorption and scattering, are very popular. The Normalized Difference Vegetation Index (NDVI) is amongst the most used. The potential for using NDVI or related indices to predict biomass and grain yield has been reported in wheat under water and heat stressed conditions [61-64], maize [65-68], sugarcane [69] and sorghum [70]. In most of the papers cited, different nitrogen regimes were imposed to amplify the response range and avoid saturation of the index, facilitating the correlations with yield. In cases evaluating genotypic variation, the correlations between NDVI and grain yield have been established both during grain filling period, associated with canopy or flag leaf senescence onset or rate [64, 71] or early canopy development [72], depending on the prevalent limitation, e.g. water supply, etc. Other, useful indices, are based on regions in the NIR that specifically relate to tissue water content, such as the Water Index (WI) and/or normalized water index (NWI) that assesses the water content of the canopy [73]. Yield predictions using water indices have been observed not only under water-stressed but under well-irrigated conditions and high-temperature environments in wheat [58, 60, 74-76], drought and irrigated conditions in barley [77]; different drought regimes in maize [78, 79] and moderate to severe water deficits in vineyards [80, 81]. It has been stated that water indices often have a higher ability than NDVI to predict yield across water stress scenarios [60, 82]. More recently,

models including all available hyperspectral reflectance bands derived with point or imaging sensors have been validated as predictors of yield in wheat [83], rice [84] and maize [78, 85], demonstrating that higher prediction accuracy can be achieved compared to using individual vegetation indices. For this type of information to become useful in a breeding context, the data processing pipeline and analysis platform need to be streamlined, currently a bottleneck for spectral information more complex than simple spectral indices.

Canopy temperature, measured with infrared thermometers or thermal imaging, is correlated to stomatal conductance, as a major determinant of the leaf temperature is the rate of transpiration, and on occasion evaporation, from leaves and other organs. Abiotic or biotic stresses often result in decreased rates of photosynthesis and transpiration and, the remote or proximal sensing of the leaf temperature by thermal imaging can be a reliable way to detect changes in the physiological status of plants regarding water use. Canopy temperature, which can be sensed remotely using IR thermometry, was shown to be highly associated with the yield of wheat cultivars, mainly under abiotic stress but also under potential conditions when evaporative demand is high [86, 87]. Canopy temperature mapped to a similar genomic region than yield in a study with a wheat population grown in contrasting stress environments [72]. A recent analysis of suitability of different platforms for application of thermal sensing in breeding trials showed that a sensor mounted on an unmanned aerial vehicle resulted in higher correlations with yield and biomass than proximal sensing [62].

4.2 Phenotyping yield components and underlying traits

Yield is generally described as the product of the grain number per unit area (e.g. grains m^{-2}) and individual grain weight or via biomass production and harvest index. Here we focus on two different approaches to directly or indirectly phenotype grain number and weight. The direct approach is based on phenotyping the numerical components per se (Figure 1a). The second one is based on an established physiological framework where growth and partitioning to reproductive structures are underlying traits that can be targeted for phenotyping the potential for diversity in grain number and grain weight formation [88, 89] (Figure 1b). In the numerical approach, grains m^{-2} can be considered a product of other numerical subcomponents formed from planting onwards (Figure 1a). The growth approach is based on evidence that in cereals such as wheat, carbon partitioning during stem elongation, a few weeks before flowering, has a strong influence on the final number of fertile florets, as the reproductive sink competes with the growing stem for carbon supply and is associated with the

number of grains m^{-2} [90]. On the other hand, carbohydrate production and partitioning after flowering influences the rate of grain growth and its final size [91, 92]. Therefore, the partitioning of carbon to reproductive structures is directly influencing the success of the numerical components of yield [93] (Figure 1b). The same framework can be applied to legumes such as chickpea, except that the critical period for biomass and grain number formation is after flowering [94]. This section describes potential pathways for phenotyping each of them, with emphasis on the determination of grains m^{-2} and grain weight, using mainly wheat but also rice as examples.

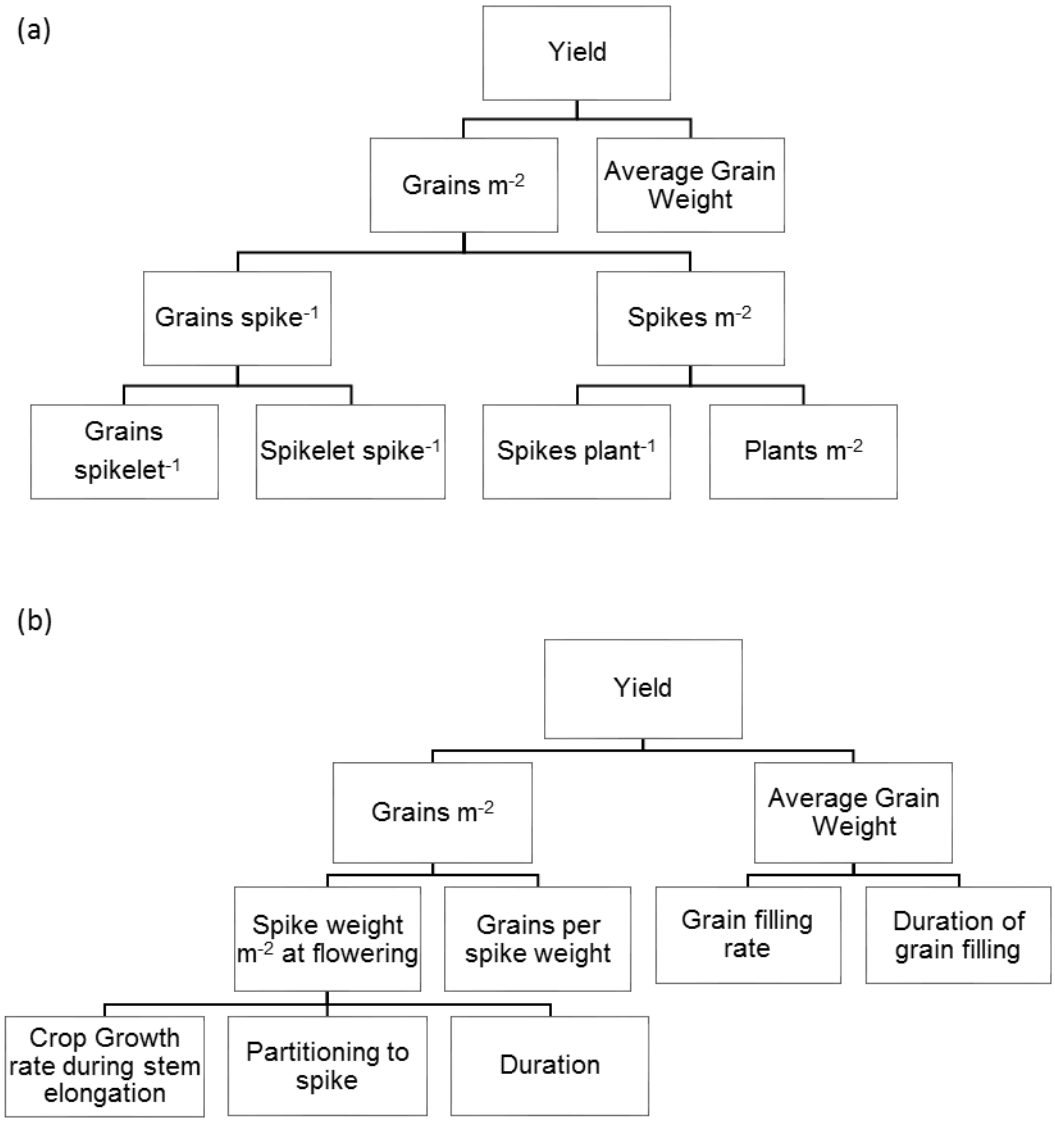

Figure 1. Diagram of yield formation in wheat (a) numerical component approach, (b) crop growth approach.

From sowing, the first numerical component in the chain is the number of plants m^{-2} (Figure 1a), which can be currently assessed with RGB images taken from ground or aerial platforms and amenable to automation in terms of data extraction [95, 96]. In pot grown plants, the number of rice panicles has been counted by analysing colour images taken from multiple angles [97]. In the field, the number of inflorescences (spikes, panicles) could be estimated from RGB images at flowering [32, 34], as mentioned in section 2.2. The use of Light Detection and Ranging (LiDAR) is currently under development for the detection of wheat ears in the field, and recent studies report an average detection rate of 85%, aggregated over different flowering stages [98]. In most crops, the rest of the numerical components are best estimated or derived from measurements after harvest, some of them object of trivial image analysis, such as grain counting or grain dimensions, which can be carried out with flatbed scanners and free firmware. What is interesting about this area is the possibility of fast-tracking sample processing with integrated approaches. A prototype with threshers and line scan cameras built for rice allows to evaluate panicle yield, number of total spikelets, number of filled spikelets, grain length, grain width, and 1000-grain weight in a single operation [99]. Other compound yield traits, such as the seed set rate and the length:width ratio can also be derived from the extracted traits. This prototype comprises three major units: the threshing unit, the inspection unit, and the packing and weighing unit (Figure 2a). The mean absolute percentage error was less than 5% for all of the evaluated yield traits, and the efficiency was approximately 1440 plants per 24-hours continuous workday. Simultaneous grain dimension, visual characteristics and composition analysis have also benefitted from advances in imaging, robotics and classification techniques. There are numerous lab based scanner platforms with integrated software capable of counting, analysing and sorting single grains based on colour, vitreousness or other properties with high efficiency, e.g. 1000 kernels per minute with Satake 'RSQI10A Grain Scanner' (http://satake.com.au/lab/Grain_Scanner.htm). Instruments exploiting NIR features, e.g. transmission, such as Next Instruments 'CropScan Automated Grain Analysis System' (http://nextinstruments.net/index.php/products/cropscan) can provide additional information on protein, fats and oils, water and carbohydrates from specific calibration models. With increased automation and speed, platforms such as QSorter Explorer, which can process 50 grains per second, develop a NIR spectrum and 3D image and sort or classify grains based on shape, composition (protein, oil, amylose content and other properties) and other properties (Figure 2b).

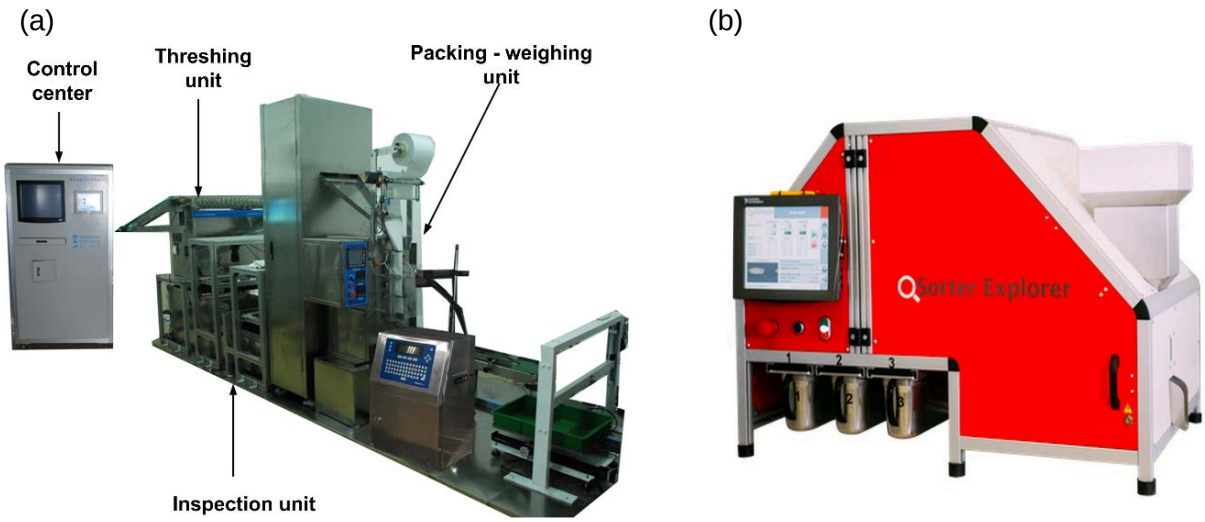

Figure 2. Examples of integrated instrumentation to evaluate multiple traits: (a) prototype built for rice yield component analysis [99] (b) QSorter Explorer platform for grain analysis.

There are increasing prospects of phenotyping some of the components of the resource-oriented model (Figure 1b). The possibility of estimating the evolution of crop biomass with a LiDAR based system before or after flowering [100] means that estimates of crop growth rate and fraction intercepted are closer to being high throughput in the field, facilitating the derivation of radiation use efficiency. In crops where the inflorescence is exerted, it may be possible to distinguish spike or panicle growth and potentially grain filling rate, as opposed to measuring crop growth rate, which may include biomass deposition in non-grain organs under certain circumstances. An additional source of carbon for grain growth during grain filling in wheat and barley is the mobilisation of water soluble carbohydrates (WSC) from stems and sheaths to grains [101]. The accumulation of WSC (mainly fructans) in stems starts up to 20 days before anthesis and continues during most of grain filling with the potential of attaining more than 40% of the stem dry weight in wheat [102, 103]. The concentration of WSC in stems and/or sheaths is comonly measured using spectra-photometrical methods, such as anthrone [104] or NIR reflectance spectroscopy [105]. Another non-high throughput method to estimate WSC content is monitoring post-flowering changes in stem dry weight due to the strong correlations between these two traits under field conditions [106]. These methods always involve time consuming samplings and sample preparations (e.g. drying, milling, weighing). Using a different high throughput approach, the dynamics of stem WSC was robustly predicted (concentration and amount per unit area) in wheat with hyperspectral reflectance, although further investigation was suggested to

limit the model to bands below 1000 nm to be able to rely on cheap detectors [107]. An added benefit of estimating WSC concentration with reflectance is the possibility to extract other data such as leaf area index and canopy water content from the same measurements [107]. In practice, the development of high throughput methods for WSC estimation represents one of the biggest challenges in plant phenotyping, as the small effects of many WSC QTLs may limit the use of markers in breeding programs for stem WSC [108]. Another source of carbon for the fertile florets initially and the growing growing grains after fertilisation is spike photosynthesis [109], the complexity of measuring spike volume to help in the quantification of photosynthesis was discussed in Box 1 (Molero, unpublished).

Box 1. Inflorescence morphology and area

Inflorescences dominate crop canopies from flowering onwards. They intercept radiation, photosynthesise and can mobilise reserves [110, 111] in addition to their primary function of bearing grains (wheat and barley spikes, rice panicles, maize ears, canola racemes, etc) or act as pollen reservoirs (maize). In recent years, there has been a resurgence in the interest in morphology and function of inflorescences, their genetic regulation and how they influence yield and its components. In rice, changes in several candidate genes have been shown to regulate panicle architecture and grain number [112, 113]. Most of these studies were based on pot plants measured manually, only recently a phenotyping imaging pipeline that allows the characterisation of many panicle traits simultaneously (panicle and branch length, internode and rachis length, branch number and order, etc) has made possible the characterisation of a large germplasm collection for genome wide association studies [114, 115]. In comparison to rice, characterisation of the wheat spike in a high throughput system lags behind, despite the interest in the regulation of its morphology [116-118]. High throughput estimates of spike area or volume, weight and spikelet number would unlock access to other important traits, such as spike photosynthesis [119], fruiting efficiency [120], infertile or low grain size spikelets and following the progress of spike volume or biomass after flowering to infer grain filling rate.

When fully developed, the wheat spike consists of the main rachis with each internode ovoid in section and curving around the spikelet, presenting a vertical gradient of width [121]. Due to the spike complex three-dimensional structure and the presence of awns, the determination of its area represents a challenge, especially non-destructively in the field and aiming for high throughput. Successful approximations of spike area and volume have been reported only under controlled environment conditions with no high-throughput results that can be applied in the field [122, 123]. More recently, we used an electromagnetic 3-D digitiser (3Space Fastrak, Polhemus, Colchester, VT, USA) to estimate the area of 35 wheat spikes harvested from the field. Unfortunately, in order to calculate the spike area, the smoothing surface was increased to 1.5 mm losing the detection of the awns (Figure 3a). From the same spikes, the projected area was measured with an area meter (LI3050A/4, Li-COR, Lincoln, Nebraska, USA). In addition, the total spike area was calculated as the volume of a cylinder (Figure 3b), which showed to be representative of the spike area measured with

the area meter (Figure 3c). On the other hand, spike dry weight appeared to be a valid method to represent spike area (Figure 3d). The spike area calculated as the volume of a cylinder and that obtained with an area meter were highly correlated with the area given by a 3-D laser scanner (r = 0.89 and r = 0.90, respectively). However, only the values for spike area obtained with the area meter were similar in range to the 3-D scanner due to the overestimation using the cylinder approximation. Unfortunately, these approaches are destructive as it is necessary to harvest the spike and conduct the measurements in the laboratory implying a large investment of time and resources. As mentioned above, the development of LiDAR technologies (currently in development for counting spike number) could be applied to estimate spike volume or area as well as the use of multiple RGB pictures that would allow the 3D reconstruction in the field and, therefore, better estimations of spike photosynthesis per unit of area.

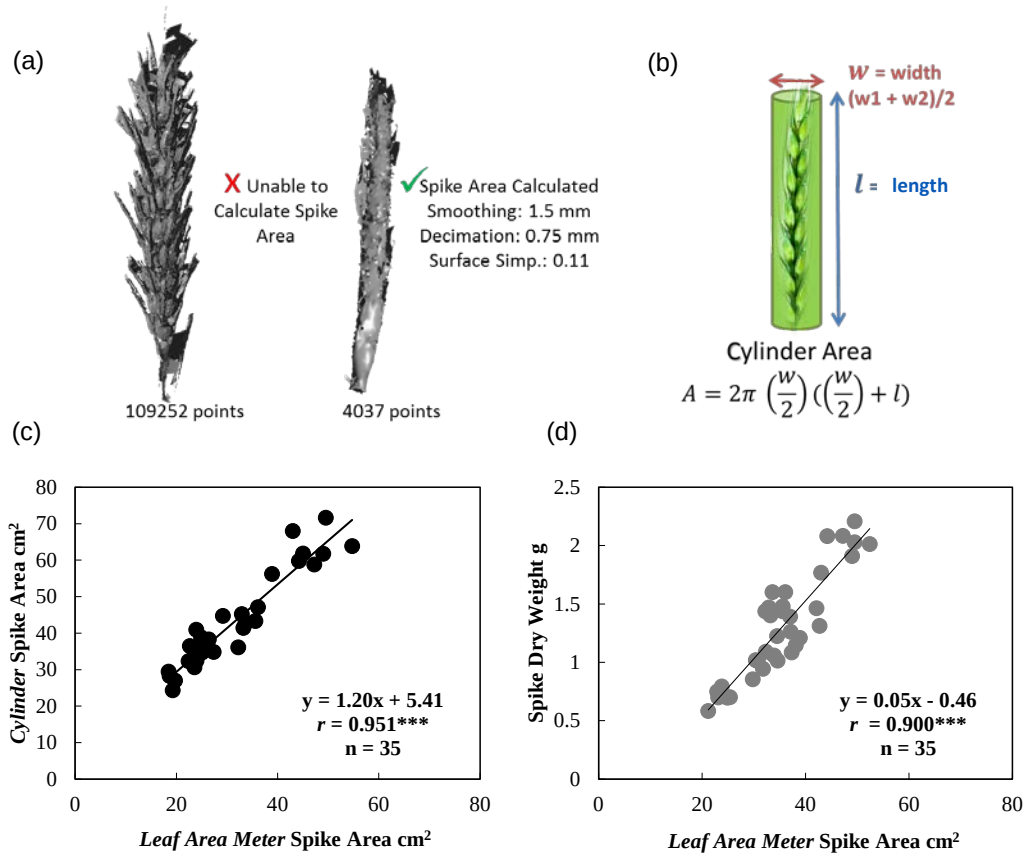

Figure 3. (a) three-dimensional spike images obtained with the FastScan scanner, using two different configurations, (b) calculation of spike area as a cylinder, relationship between spike area calculated with an area meter and (c) the cylinder approximation or (d) spike dry weight.

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5. Feasibility, impact and conclusions

The agricultural and food industries are faced with many challenges in terms of producing more food with lower environmental footprint. The decreasing cost of cameras and sensors and the parallel increase in computing power and accessibility to it, means that previously prohibiting technologies are now viable options that can add value to the plant breeding and agronomy sectors.

At this point, due to the complexity and the large number of associated traits with reproductive development, it is important to gauge the technical feasibility and the expected impact of the traits considered in this review. Both criteria, impact and feasibility, were judged based on knowledge of plant breeding phenotyping bottlenecks (impact) and the technical possibilities summarised in this study (feasibility). Highly feasible, technically easier solutions will generally be resolved with image analysis, and can potentially characterise traits with intermediate to high impact in a breeding contex, such as spikelet number, anther extrusion and colour or grain dimensions. Phenotyping solutions of intermediate feasibility will likely rely on a combination of available techniques such as RGB and hyperspectral images and/or LiDAR and target traits such inflorescence growth rate during grain filling, phenology, spike number and spike architecture. Lastly, there is a range of solutions that will rely on greater complexity but have a high impact potential such as high throuput phenotyping of internal stages or pollen viability. For the plant breeding industry, there are at least four main areas of impact for high throughput phenotyping of reproductive structures, in activities at different ends of the breeding spectrum: mining genetic resources, early generation selection, hybrid breeding and large scale field evaluation, in particular to pair with genomic information. Germplasm collections and early generations from breeding programs have in common the small amount of available seed and the large variation amongst entries. In the case of early generation selection, reducing the number of lines for further evaluation at the plot level and in multiple environments would certainly be a cost saver for breeding programs. The usefulness of this approach has already been successfully demonstrated developing germplasm for dry environments based on selection for canopy temperature [4] but would benefit from enrichment for beneficial reproductive traits. In this case, well defined, specific reproductive traits of interest should be preferably independent from growth, not likely to be obliterated when evaluated at the canopy level after crossing or further multiplication. Examples in this category from the traits reviewed in this study are phenology and generation of specific yield components, such as spikelets per spike in wheat, inflorescence morphology and branching, pollen and anther related traits and dry

matter partitioning to the inflorescence. The positive association between spike to biomass ratio at anthesis measured destructively in single plants and crop yield was promising in durum wheat [124] and it would be worth exploring the suitability of LiDAR based phenotyping for this trait as well as spike growth rate during grain filling at the canopy level, in relation to increase in volume. Both targets would be of medium to high impact in breeding and intermediate feasibility, based on what we know about other LiDAR applications [100]. With regards to hybrid breeding, in field assessment of pollen shedding and mass and anther extrusion, key traits for the male ideotype [48], could clearly benefit from image analysis technologies or even spectral applications, with potentially high impact and easy to intermediate feasibility. Lastly, amongst the field based breeding efforts, genomic selection is one of the most likely areas to benefit from large scale phenotyping of reproductive traits to capture environment dependent allelic variation. Genomic selection uses all the available molecular markers across the entire genome to estimate genetic or breeding values, based on a training and validation data sets. A training data set that has been phenotyped and genotyped is used to calibrate a prediction model, which is then used to predict the breeding values of the test set of genotyped selection candidates [125]. Traits measured using high-throughput phenotyping based on proximal or remote sensing, such as canopy temperature and vegetation indices, improved pedigree and genomic prediction model accuracies for grain yield in wheat [3]. The consideration of novel traits related with reproductive growth will not only increase the accuracy of the models and assist towards faster and greater genetic progress, but also enrich the model in an area directly relevant to yield.

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