

Review

Sensing Technologies for Precision Phenotyping in Vegetable Crops: Current Status and Future Challenges

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Abstract: Increasing the ability to investigate plant functions and structure through non-invasive methods with high accuracy has become a major target in plant breeding and precision agriculture. Emerging approaches in plant phenotyping play a key role in unraveling quantitative traits responsible for growth, production, quality, and resistance to various stresses. Beyond fully automatic phenotyping systems, several promising technologies can help accurately characterize a wide range of plant traits at affordable costs and with high-throughput. In this review, we revisit the principles of proximal and remote sensing, describing the application of non-invasive devices for precision phenotyping applied to the protected horticulture. Potentiality and constraints of big data management and integration with “omics” disciplines will also be discussed.

Keywords: digital imaging; genomics; phenomics; plant breeding; greenhouse horticulture; advanced crop management; automation; vegetation indices; optical sensors; fluorescence

1. Introduction

Modern agriculture in the next decades will face several challenges related to the rapid increase of world population, climate changes, the need for more sustainable and environmental friendly cultivations, and the growth in interest for high food quality and safety by producers and consumers. To address these problems, an optimal management of resources, in concert with innovative methods to increase the performances of crops in terms of yield and resistances to biotic and abiotic stresses, will be necessary. To this end, precision phenotyping—aimed to enhance the assessment of crop traits and selection of improved genotypes—is one of the main targets for geneticists and breeders. In the last 150 years, fundamental milestones have been reached in the field of genetics and genomics. The knowledge of inheritance theories by Mendel and the mechanisms of linkage and recombination in plants defined the association between genotype and phenotype, laying the foundations for studying complex traits. Afterwards, advancements in molecular biology as well the development of next generation sequencing technologies have made strong contributions to the investigation of crop genomes and the understanding of gene function. The increase of the throughput for nucleic acids investigation and the related reduction in costs made these systems accessible to a wide community, helping the dissection of genetic diversity and its utilization toward the introduction of desirable alleles, enhancement, and precision breeding. Despite the plethora of genomic information gained from genotyping, transcriptome, and sequencing studies in plants, comprehensive phenotypic characterization in plants has not yet been achieved. High processivity and quality in phenotyping is labor intensive, requiring technologies, competences, and assets not accessible to all research groups.

This aspect, better known as “phenotyping bottleneck” [1], poses the risk of an underutilization of the progress achieved in sequencing and of the potentiality stored in genetic resources. In fact, it is well known that, for a large number of genes, the function is still unknown; moreover, for the wide experimental mapping populations and collections established in many crops, the lack in phenomics knowledge reduces the potentiality of these resources as sources of traits for genetic improvement.

The identification of genotypes carrying superior traits of agricultural interest has ever been the primary target for genetic improvement of crops. Historically, plant breeders evaluated, across environments and years, large sets of genetic materials, segregant populations, and various crosses. The process leads to selecting the best-performing individuals and identifying traits to be introgressed into agronomic backgrounds through recombination breeding only on the basis of the visible phenotype. Afterwards, marker-assisted selection (MAS) has sped up such processes, becoming a routine procedure in many breeding programs and providing a direct way to select traits associated to relevant DNA sequences. In many species, the basis for MAS have been provided by the development of genetic maps and linkage disequilibrium (LD) studies [2,3]. These strategies—which are based on the association between a genotyped marker and a phenotype of interest, evaluated in a large number of individuals—have allowed resolving the genetic architecture of many characters, identifying markers tightly linked to genes and QTLs (quantitative trait loci). The development of robust DNA-based markers is also essential for efficient and successful marker-assisted crop breeding programs. From the genetic point of view, the reliability of a molecular marker to predict the phenotype is related to the strength of the linkage with the relevant gene, which, in the case of linkage disequilibrium, depends on the model used to discover associations, while linkage mapping is affected by recombination events. From the phenotypic perspective, genetic associations depend on the accuracy of the estimation of a desired character. The better the phenotyping, the greater the chance to get precise associations. It must be considered that most of the traits of agronomic interest are quantitative, requiring extensive efforts for their assessment. While conventional methods perform well for qualitative traits, they have been slow to improve the effectiveness of understanding complex traits. As a result, despite the hundreds of published studies, several QTLs are not reliable for applied breeding due to an inaccurate estimation of the underlying traits, which is one of the causes of the discovery of false associations. Therefore, precise phenotyping with high-throughput tools is imperative in order to dissect the genetic and environmental components affecting complex traits. To this end, the scientific community has increased interest in the application of remote sensing technologies, leading to the development of a number of techniques and methodologies able to characterize morphoagronomic, physiological, and metabolic traits in crops. It has been estimated that, in the next decade, the development of well-performing phenotyping strategies that are able to increase throughput and reduce costs will mirror the innovation in genomics that has occurred in the last 20 years, laying the foundation of the next era of digital science [4].

2. Approaches for Phenotyping

Phenotyping can be performed at different depth scales (high and low resolution) and processivity (high and low throughput) [5]. High-throughput techniques in general involve the analysis of the whole plant with medium-low resolution. This is the case of the technologies implemented in field phenotyping for resource management and crop monitoring—such as nutrient and water distribution, weed infestation, and epidemiology—which allow the screening of hundreds of plants per day in a nondestructive manner with automated systems [5]. High resolution is instead applied for both investigations at plant organs or whole plant levels as well as at cellular level. The latter requires invasive methods (i.e., metabolomics) that are not fully automatable due to the prior steps required for sample preparation, and a throughput generally low [5]. On the basis of the procedures for data acquisition, mobile or stable systems have been designed. In the former case, which is commonly applied in open field, plants are fixed and the station moves while measuring traits. In the second case, plants are instead transported to the imaging station. This system is generally applied in indoor

facilities such as growth chambers or greenhouses [6]. High-throughput phenotyping platforms (HTTPs), able to measure plant architecture, growth, and environmental parameters through the combination of different devices for imaging analysis and data processing, have been developed in the recent years in different research institutes such as INRA (PhenoArch, Phenopsis, and Phenodyn), FZ Jülich (Jülich Plant Phenotyping Center), IPK (Leibniz-Institute of Plant Genetics and Crop Plant Research), and HRPPC-CSIRO (The High Resolution Plant Phenomics Center), or are currently sold by private companies (e.g., Lemnatec, Phenospex) [7–11]. Moreover, international (IPPN, International Plant Phenotyping Network; EPPN, European Plant Phenotyping Network) and national efforts (i.e., DPPN, German Plant Phenotyping Network; ITA PPN, Italian Plant Phenotyping Network; APPE, Australian Plant Phenomics Facility, etc.) are ongoing in order to establish joint strategies for large-scale phenotyping and to facilitate networking in order to share the knowledge. These abovementioned platforms can be implemented in protected agriculture as well as in open field. Field phenotyping, although informative, needs to take into account the heterogeneity of the soil and the climatic conditions, which complicate the analysis and interpretation of data. On the contrary, the information obtained in controlled conditions are difficult to translate in the field, in particular for those traits under strong influence of the growth environment, such as yield and quality. For this reason, the development of performing research on tools for data analysis and integration is underway as a priority target. Pioneering studies using advanced phenotyping platforms have been mainly conducted in model species such as *Arabidopsis* and extensive crops such as cereals and oleaginous [12]. However, minor efforts have been carried out in vegetables. Thanks to the large number of uses and the added value given to the diets, vegetable production plays a major role in modern society and global economy. The impact that innovative phenomic strategies could have for the improvement of the vegetable crops chain could therefore be substantial. Advanced plant phenotyping platforms, although performing well, are quite costly in terms of investments, data acquisition and handling, management across environments, and needs for specialized personnel. Therefore, these platforms are not easily affordable in many researches. Taking into account that the protected environments, rather than the open field, are the systems of cultivation largely used in vegetable crops, it is possible to consider simple and cost-efficient methods with high-throughput capacities for phenotyping. The aim of this review is to describe the applications of nondestructive sensing technologies for high-throughput phenotyping, giving an outlook of their role in plant breeding to unravel quantitative traits responsible for growth, production, quality, and resistance to various stresses. Descriptions of HTTPs and indoor facilities, including those involving destructive techniques (i.e., metabolic studies), are beyond the scope of this review, since they have been already accurately described in other reports [6,7]. Phenotyping studies carried out in vegetables, concerns, and future perspectives of big data management will be discussed.

3. Proximal and Remote Sensing Technologies in Horticulture

3.1. Definition and Reference Characteristics

Much of the work traditionally done by human beings in plant breeding can be accomplished by electronic devices, an outcome of the “computer era”. In phenotyping, many high-tech tools can offer worthy solutions for high-precision and fast characterization of plant material. Hand-held and robot-mounted proximal and remote sensors (PRSs) are, for this purpose, available for detection of: (i) plant structure and morphogenetic traits; (ii) abiotic and biotic stresses; and (iii) elemental and molecular characteristics. The main advantages in the adoption of these tools for high-throughput phenotyping consist of: (i) collection of huge quantities of data and their safe storage; (ii) very fast and well-organized working flow; (iii) economical and time-saving analysis procedures; and (iv) collection of objective data (i.e., not influenced by human perception), which can be handled by different linked users. Therefore, the use of such kinds of technologies has been increasing very rapidly in the last decade [12–15], thanks also to the increased availability of microcomputers (including laptops, tablets, and smartphone) and internet/intranet and wireless connections [16].

The background idea in the adoption of PRS-based technologies for plant monitoring lies in the concept of “speaking plant” according to which plants are themselves revelatory of their own physiological status detectable through PRS measurements [17,18]. Many PRS-based monitoring systems, successfully applied in phenotyping studies, very often were originally developed or planned for other scopes in horticulture. In fact, many devices come, or are derived, from other application areas that mostly regard crop management—in particular, plant pathogen defence and plant nutrition and irrigation [15,18,19]—other than plant physiology.

The use of PRSs appears reliable and effective for both crop management and phenotyping. However, most indices calculated through PRSs are nonspecific, thus revealing information on plant status that could be caused by different internal-to-plant and external (environmental) variables. For instance, to assess plant stress, chlorophyll content and the status of the photosynthetic machinery are often monitored through different spectroscopy methods using PRSs. Chlorophyll content and photosynthesis can be affected by a plethora of biotic and abiotic stresses, all signalled in plant metabolism by hormones, such as ABA (abscisic acid), that cause stomatal closure and reduced photosynthetic activity [20]. For example, drought and salinity can induce the same effects on stomatal conductance and photosynthesis, while the shortage of many different nutrients can induce reduced chlorophyll content in leaves to different extents [20,21]. In the above scenario, it is sometimes difficult to discern which agronomic variable is really influencing the plant status at the end of the crop operative management, and even more difficult is converting the stress signal to practical applications to restore optimal growing conditions (e.g., quantity of fertilizers and water or pesticide, optimal temperature and radiation level, etc.). However, there are examples of efficient crop management based on sensor applications [18]. The use of PRSs for plant phenotyping appears quite effective in achieving the scope of genotypic selection, where the variables influencing the observed plant performances are artificially controlled and, therefore, nonspecific physiological responses can be directly linked to specific treatments.

Optical and thermal sensors are the most widely used tools in plant phenotyping. Many sensors fall in this category; however, only those devices that allow high-throughput phenotyping will be discussed in the present paper, while other instruments will only marginally be reported. The working principles of PRSs are mostly based on the measurement and analysis of electromagnetic radiation (spectroscopy) that can vary as a function of its interaction with plant tissues. However, PRSs can be classified considering many different characteristics. Depending on the spatial acquisition of detected data, PRSs can roughly be divided into the categories of point sensors (e.g., spectroradiometers and fluorimeters) and imaging sensors that allow the acquisition of spatial information of the detected data [22]. Imaging techniques indeed represent, among others, a large portion of PRS-based measurements in phenotyping, and their applications cover a wide range of different types of observations for the estimation of many biometric and physiological parameters [14,15,23]. Imaging phenotyping can be accomplished through the simple acquisition of 2D images or by 3D models generated by multi-perspective acquisitions [24] and 3D images created by laser-scanner imagers [25,26]. With respect to the electromagnetic spectrum, measurements can be done in the range of ultraviolet (UV), visible (VIS), near-infrared (NIR), and infrared (IR) radiation [11], each providing different information. The broader the covered wavelength range and the number of measured wavelengths, the higher the detection capability. Therefore, instruments working in the hyperspectral (from tens to hundreds of wavelengths) range offer more flexibility than multispectral (from two to tens of wavelengths) analysis or single-wavelength measurements. On the other hand, the broader the band range measured around a specific wavelength, the lower the measurement accuracy due to the overlap of different wavelengths, even if some indices will be more stable when calculated in broader bands [27].

For a better understanding of how PRS technologies can match breeders’ needs in vegetable crop phenotyping, it appears more useful to describe the different PRSs, taking into account their working principles relative to the way in which plants interact with the electromagnetic radiation.

3.2. Radiation Interception

Measuring the quantity of radiation (e.g., photosynthetically active radiation (PAR) or global radiation) intercepted by plants is one of the simplest operation modi of PRSs. It is based on the principle that any fluid, solid, or gaseous entity interferes with electromagnetic radiation by simple interception. Measurements based on radiation interception are commonly applied in the detection of plant organ morphology and canopy architecture characterization, plant biomass and volume estimation, and in the calculation of different canopy indices such as the leaf area index (LAI) [14,15].

The simple measurement of light extinction—through the positioning of two PAR sensors above and below the canopy (e.g., by using a ceptometer)—has been found to be sufficient for the estimation of crop LAI [28]. Digital cameras in the range of VIS bands (400–700 nm) allow capturing 2D images in which raw data are spatially recorded in the red (≈ 600 nm), green (≈ 550 nm), and blue (≈ 450 nm) array by a CCD (charge-coupled device) or CMOS (complementary metal oxide semiconductor) silicon-made sensors. This kind of PRS can be used for the estimation of plant biomass [29], leaf area and LAI [30,31], and plant morphology [32,33].

However, 2D images present many limitations, especially when used for plants that have a high degree of structure complexity, therefore, 3D images are preferred [26]. The use of stereo camera rigs and the analysis by computer programs of images taken by multiple angulations allow drawing sophisticated models for the reconstruction of plant structures in 3D [24,34]. Yet, digital cameras offer further characteristics that deal with plant color analysis, which is later addressed in the text.

Indeed, a number of PRSs can accomplish multiple functions in plant phenotyping, including 3D imaging, however, to the specific purpose of plant structure and biomass analysis, the most widely adopted technologies are based on light detection and ranging (LiDAR) by using laser-scanner sensors [35,36]. LiDAR technologies consist of an active laser sensor providing direct measurements of canopy architecture and organ distribution [25] for the estimation of plant volume, LAI, and biomass [25,35,37,38], thus allowing plant growth analyses from the vegetative to reproductive stages [26,39,40]. Other techniques include time-of-flight cameras and ultrasonic sensors, reviewed in [14].

3.3. Radiation Reflectance, Absorbance, and Transmittance

Plant spectral response analysis is one of the most effective, meaningful, and widely used approaches for high-throughput phenotyping. The interaction between radiation and plant leaves—in terms of spectral reflectance, absorbance, and transmittance—can be defined as a passive process in which incoming electromagnetic radiation is affected by the relative quantity, but not in the wavelength form (as occurs, for example, in the fluorescence phenomenon). Reflectance, absorbance, and transmittance measurements can be related to plant structural and chemical characteristics [31,41–43], thereby allowing the assessment of plant water and nutrient status [31,43,44] and photosynthetic activity, the detection of biotic and abiotic stress [25,27], and the evaluation of different plant physiological statuses [26,27,45]. In fact, reflectance, absorbance, and transmittance of electromagnetic radiation are influenced by plant tissue morphology and elemental and molecular composition [15]. Digital cameras, as previously described, can be adopted to capture images in the VIS range, providing useful information on plant color, for the detection of plant stress and plant classification, in addition to structural and biometric characteristics [38–40,46–50]. However, PRSs based on reflectance measurement can provide more useful information in plant physiology studies. Multispectral imaging consists of measuring leaf reflectance at different wavelengths, thus providing data for the calculation of useful vegetation indices [15,51] (Table 1). Basic multispectral cameras are equipped with RGN sensors, where N stands for near infrared (NIR), while in the more sophisticated types, the acquisition of multiple NIR wavelengths is coupled with multiple measurements within the VIS bands. As a basic principle, plants showing higher reflectance in the NIR bands (≈ 700 – 1000 nm) and lower reflectance in the PAR regions are performing better than plants showing the opposite trend [15,23,52]. Examples of cameras commonly used in experimental works are Tetracam ADC Snap NRG camera (Tetracam, Inc., Gainesville, FL, USA) and MicaSense RedEdge-M (MicaSense, Inc., Seattle, WA, USA) [41,53].

The main disadvantage with multispectral camera acquisitions in the VIS/NIR bands lies in the fact that the sensors are sensitive to a quite broad spectrum of electromagnetic radiation, therefore, specific information from single wavelengths are not detectable. Canopy evaluation in the VIS/NIR range are in fact often based on nonspecific plant physiological responses, that is, mostly leaf photosynthesis and chlorophyll concentration, since chlorophyll of healthy leaves commonly absorbs 70–90% of the light in the PAR regions [15,46]. Such a drawback indeed appears more relevant in the use of PRSs for crop management than phenotyping, where stress variables are induced ad hoc and their related plant symptoms are often known in advance. It should also be stressed that reflectance properties change much among different organs or organ status (age, stress conditions), therefore, it is not possible to calculate generalized indices; for example, fruits at ripening usually show trends much different from leaves [49]. These limitations can, however, be overcome by the acquisition of the electromagnetic spectrum in the shortwave infrared regions (SIR). The sensors of NIR/SIR cameras adopt technologies different from silicon (e.g., indium–gallium–arsenide) that allow measurements in the range of 900–1700 nm where, for example, water absorption regions are present for water indices estimation [23]. For this scope, hyperspectral cameras are powerful PRSs performing at high resolution (i.e., 1–5 nm) and measuring hundreds of bands in the spectral range between 350 and 2500 nm [14,23]. In the SIR regions, biotic and abiotic stress can be detected more specifically, thereby performing cause–effect analysis at a high-precision level [42]. The use of digital (multispectral and hyperspectral) cameras allows performing 3D imaging as previously described; however, being passive PRSs, digital camera acquisition requires continuous adjustments depending on the radiation source [14,23].

Alternatively, spectroradiometers are valuable instruments for phenotyping purposes [22,43,53] that have a high capacity of acquisition in very broad ranges of electromagnetic radiation (350–2500 nm). Spectroradiometers do not allow the acquisition of 2D or 3D images, but are very powerful optical sensors that offer many advantages such as high band definition, portability, spectrum range, and data handling. The last aspect, for example, is one of the main concerns (i.e., data dimensionality) in the use of hyperspectral cameras (other than the very high cost) [23]. Spectroradiometers are available as both passive and active PRSs [19]; the latter allow measurements by active irradiation of the subject, thus obtaining more comparable data not dependent on an external (environmental) radiation source. Alternatively, the measurement with passive sensors can be normalized through the transformation of the incoming radiation recorded simultaneously with reflectance measurements [22]. Examples of passive and active sensors are CropScan® (Cropscan, Rochester, Nebraska, NE, USA) and FieldSpec® (ASD PANalytical, Boulder, Colorado, CO, USA) or Crop Circle® (Holland Scientific, Lincoln, NE, USA) and GreenSeeker® (Trimble, Sunnyvale, California, CA, USA), respectively [19]. Data collected through hyperspectral cameras and spectroradiometers can be analyzed using the same indices adopted for multispectral analysis.

3.4. Fluorescence

As analytic methodology, fluorescence analysis belongs to spectroscopy but differs greatly from reflectance, absorbance, and transmittance measurements in the way by which plant tissues interact with electromagnetic radiation. The fluorescence phenomenon is based on the active interaction between plant tissue and light—the electromagnetic radiation, initially absorbed by the photosynthetic machinery as energy source (i.e., photosystem excitation usually induced through a beam of light in the blue or red wavelength band of visible spectra) [44] is then reemitted at wavelengths that are different from those absorbed. Blue-green (400–600 nm) and chlorophyll (650–800 nm) fluorescence can both be detected in the fluorescence analysis of green leaves [14,15]. Data collected by fluorescence spectroscopy can therefore be related to leaf chlorophyll content and photosynthesis activity. Compared with spectral analysis (i.e., by digital cameras or spectroradiometers), fluorescence spectroscopy can provide detailed information about leaf photosynthetic efficiency through the measurement of photochemical and nonphotochemical quenching, in addition to the maximum quantum efficiency of photosystem II (F_v/F_m). The last parameter can be calculated as a function of the maximum

fluorescence recorded after a saturating light pulse in the red region and the minimal fluorescence value recorded at a low-light induction level on dark-adapted leaves [23]. Fluorescence measurements can be conventionally grouped as follows: (i) room-temperature fluorescence; (ii) low-temperature fluorescence; (iii) fluorescence temperature curve; (iv) variable chlorophyll fluorescence [44]. The latter can be determined in different ways, however, the most diverse approaches regard the application of the fast chlorophyll fluorescence or plant efficiency analyzer (PEA) and the pulse amplitude modulation (PAM) fluorimetry [44]. Fluorescence analysis can be utilized in phenotyping of vegetable crops to assess biotic and abiotic stresses [45], tissue chemical composition and characterization [54], and different plant physiological conditions [14,49,55]. Furthermore, chlorophyll fluorescence imaging (CFI) is a step forward in fluorescence analysis, accomplished by the support of CCD cameras. In this case, different lamps are used to induce fluorescence excitation while the plant response is monitored by the digital camera measuring fluorescence at different wavelengths in the typical spectral ranges of blue (440 nm), green (520–550 nm), red (690 nm), far-red (740 nm), and NIR (800 nm) [14,56].

3.5. Radiation Emission

Plants, as biological entities, actively emit electromagnetic radiation in the IR region due to tissue surface heating. This phenomenon strongly depends on water transpiration, by which plants regulate their temperature, taking advantage of the cooling effect of water evaporation occurring in the substomatal cavity. Temperature variations can then be detected by radiometers in the spectral range of longwave IR (usually 3–14 μm) [14,23]. Thermal imaging consists of the use of thermal cameras that are sensitive in the IR spectrum, allowing for thermographic representation of plant surface. Since leaf transpiration and temperature are correlated with each other, one of the most valuable uses of thermal cameras is their application in the study of plant water relations regulated by stomatal conductance [57]. However, abiotic and biotic stresses can also be detected with this technique, as direct or indirect causes of stomatal closure [58,59]. As well, other physiological aspects related to stomatal activity (e.g., photosynthesis) can be derived by thermal measurements [57]. Although very worthy in principle, the application of thermal sensors presents some limitations due to the influence that many environmental variables can have on plant surface temperature (e.g., radiation, wind, soil/substrate evaporation). To this purpose, many efforts must be made to normalize output data by using measurements in the area surrounding the observed plot [60].

3.6. Magnetic Resonance

Magnetic resonance imaging (MRI), based on nuclear magnetic resonance (NMR) analysis, allows measuring resonance signals produced from H, C, and N isotopes. With this technology, 3D acquisition can be accomplished to acquire information on plant morphology with high precision; in particular, this approach has been appreciated for imaging roots in the root zone [61]. NMR is also used for the detection of water and labeled molecule fluxes within plant organs [62–64]. Improved applications of MRI can be accomplished by combining MRI with similar techniques, such as positron emission tomography (PET) based on the measurement of gamma ray emissions [65,66].

3.7. Performance Indices

Output information provided by PRSs can eventually be used to calculate different indices of plant performance directly and/or indirectly linked to many plant characteristics and different physiological responses and to biotic and abiotic stresses, in addition to agronomic efficiency indices. Measurements of the electromagnetic radiation response, detected through PRSs at different wavelengths, are numerically transformed and combined in mathematical equations for the computation of vegetation indices. These indices can then be related to specific plant physiological or performance indicators. The highest is the degree of correlation between the observed parameters and the calculated indices, the highest is the reliability of the selected model [67]. Yet, to compare different mathematical models, indices that show linear relationships with the observed parameter are generally preferred [27].

For spectral analysis, the normalized difference vegetation index (NDVI) [52] is one of the most common and widely used index reported in scientific literature on vegetable crops. NDVI has been tested for the assessment of plant water status [68], leaf chlorophyll content [69], plant mineral nutrition [67], and plant biotic and abiotic stress [48,70,71]. In the GNDVI version, the red band is replaced by the green one [72]. The introduction of the red-edge band (RE) to the calculations has sometimes produced improvements in NDVI-related indices [73,74]. More basic indices consist of the first simplified version of NDVI or GNDVI, as in the case of the red and green vegetation index (RVI and GVI) [75]. More specific indices have been designed, for example, for leaf chlorophyll content and plant water stress [76,77].

Table 1 reports a non-exhaustive compendium of some of the most common vegetation indices that can be adopted in vegetable phenotyping. Many other new indices, or rearrangements of the old ones, are indeed available [78,79]. However, while the calculation capacity is potentially unlimited, vegetation indices are often nonspecific. Therefore, to obtain very reliable datasets, the main difficulty lies in the selection of specific wavelengths that are directly related to the monitored parameters.

Table 1. Examples of the most common vegetation indices potentially available for phenotyping studies in vegetable crops.

| Index | Common Abbreviation | Formula ^a | Crop | Reference |
|---|---------------------|---|--|---------------------------------|
| Green Normalized Difference Vegetation Index | GNDVI | $\frac{\lambda_{NIR} - \lambda_G}{\lambda_{NIR} + \lambda_G}$ | cucumber, lettuce, tomato, onion | [70,71,77,80,81] |
| Green Vegetation Index | GVI | $\frac{\lambda_{NIR}}{\lambda_G}$ | cucumber, lettuce, tomato | [67,73,81–83] |
| MERIS Terrestrial Chlorophyll Index | MTCI | $\frac{\lambda_{NIR} - \lambda_{RE}}{\lambda_{RE} - \lambda_R}$ | cucumber, lettuce | [67,71] |
| Normalized Difference Vegetation Index | NDVI | $\frac{\lambda_{NIR} - \lambda_R}{\lambda_{NIR} + \lambda_R}$ | broccoli, cabbage, cucumber, leaf mustard, lettuce, onion, pepper, radish, spinach, tomato | [42,46,48,67,69–71,79–82,84,85] |
| Red-Edge Index | REI | $\frac{\lambda_R}{\lambda_{RE}}$ | broccoli, onion | [79,80] |
| Red-Edge Normalized Difference Vegetation Index | RENDVI | $\frac{\lambda_{NIR} - \lambda_{RE}}{\lambda_{NIR} + \lambda_{RE}}$ | cucumber, tomato | [67,82] |
| Red Vegetation Index | RVI | $\frac{\lambda_{NIR}}{\lambda_R}$ | cucumber, lettuce, tomato | [46,67,71,81] |
| Water Index | WI | $\frac{\lambda_{900}}{\lambda_{970}}$ | lettuce, onion, tomato | [70,79,86] |

^a Wavelengths in the green (λ_G), near-infrared (λ_{NIR}), red (λ_R), or red-edge (λ_{RE}) spectral range.

4. Controlled Indoor Environments: From Cultivation to Phenotyping

To complete the life cycle, plants need suitable climatic conditions (air, temperature, humidity, CO₂) and water and nutrient supplies, which, if optimized, can boost production and reduce waste. The concept of controlled environment has evolved since the second half of the last century due to the spread of roofing materials, which have allowed the establishment of various types of greenhouses for different types of crops.

Thanks to the control of microclimate and the fertigation, cultivation in greenhouses can minimize the variability of both soil and aerial environments, since crops do not compete for nutrients and optimal allocation of the roots. In addition, they are not exposed directly to weather threats such as wind, rain, and cold, among others. A fine climate control allows for homogeneous crops, with minimal differences between plants, and cultivates them with defined, highly reproducible protocols for comparable data collection. The concept of controlled environment for cultivation can therefore be applied to the precise phenotyping for those traits difficult to assess and expensive to carry out in open field.

An example is the soilless culture system, in which plants grow without use of soil as a rooting medium and inorganic nutrients are supplied at appropriate concentrations in the irrigation water,

named nutrient solution. Compared to soil cultivation, pot sub-irrigation allows to greatly reduce the variability of the root environment because the substrate is homogeneous and standardized and the irrigation is specific for each pot, depending on the consumption of the plant and the capillary rise of the nutritive solution. Controlled conditions of nutritional stress can be achieved by varying the nutrient solution; for example, the nutrient solution can be enriched with specific fertilizers and/or salts to select salinity-tolerant genotypes or to study their effects on produce yield and quality. Similarly, it is possible to evaluate the tolerance to heavy metals and their assimilability by plants and then select the best genotypes. The advantages in terms of absence of soil-borne pathogens and optimal input applications are innumerable; moreover, the possibility to manage water and salinity concentration allows using these systems to phenotype and select genotypes for their tolerance/resistance to drought stress. For example, experiments have been conducted in pepper to determine the complex of genes and metabolites involved in salt stress [87]. In this case, the soilless system was used to input moderate to high salt levels during the whole crop cycle. At different stages of plant growth, plant traits (biomass and yield) and physiological characterization (gas exchanges, total water potential, and osmotic potential) were performed along with gene expression analysis and metabolic profiling. During the whole crop cycle (“shoot development”, “flowering”, and “maturity of fruit” growth stages), all the biometric and agronomic traits (leaf area, yield, fruits size and weight) decreased with high saline levels (significantly at 90–120 mM NaCl), and they were correlated with gas exchanges and plant water status. Results on molecular analysis indicated that both plant developmental stage and long-term salt treatments affect the expression of genes known to be involved in ion transport, osmolyte biosynthesis, and other stress-related functions. This approach can be applied for various types of stress to identify the related genomic regions in order to develop markers for assisted breeding.

Moreover, growing plants under greenhouse conditions in targeted air temperature and humidity ranges can facilitate the selection of plants resistant to airborne pathogens. By intervening on light factors (quantity and quality) and CO₂ concentration, productive genotypes suitable for difficult growth conditions (cold greenhouse with little light in winter) or new cultivation environments (closed greenhouses, vertical farming or space bases) can be selected. Accurate measurements of the environmental indoor conditions make it easier to determine the principal variables influencing the phenotype. To this end, various types of sensors for measuring and reporting environmental parameters can be installed in greenhouses, as extensively reviewed by Both et al. [88] (Figure 1).

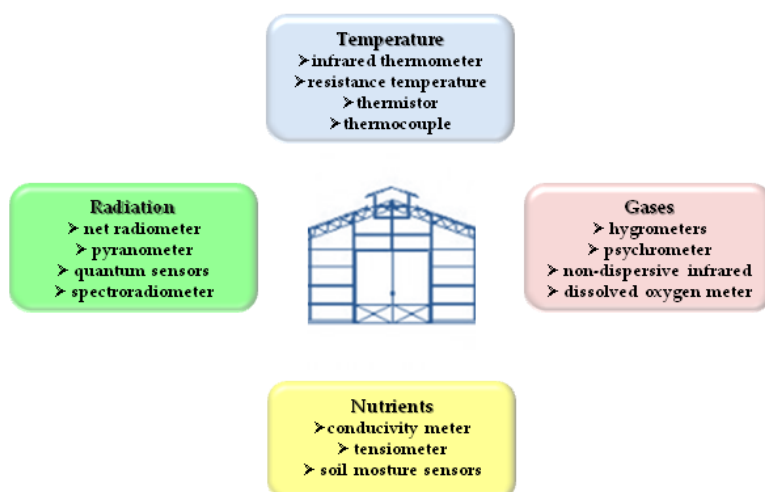


Figure 1. Sensors monitoring the environmental and nutritional conditions of plants in protected environments. Detailed description is reported in Both et al. [88].

In order to find the link between indoor and outdoor cultivation on soil, it is necessary to carry out indoor simulations of common outdoor conditions, benefitting from the lower microclimatic variability

and the greater possibility of monitoring the air and radical environment. Furthermore, the control of the microclimate and of the fertigation obtainable in greenhouse cultivations, especially in soilless crops, will increase the effectiveness of the use of nonspecific indices obtained from the PRSs because nonspecific physiological responses will be directly linked to specific treatments in ad hoc experiments. Modern plant phenotyping, plant physiology studies, and precision farming applications will benefit from this synergy.

5. Next Generation Phenotyping Studies Using Automated Devices in Vegetable Crops

Reference phenomic studies based on advanced technologies in vegetable crops are hereinafter reported with a focus on nondestructive approaches and portable devices for on-site applications (Table 2).

Table 2. List of studies performed in vegetables by means of nondestructive sensors for small-scale phenotyping and by means of portable devices.

| Plant Species | Traits | Type of Analysis ^a | Instrument | Reference |
|---------------------|---|--|--|-----------|
| Tomato and Eggplant | <i>Alternaria solani</i> and <i>Epilachna vigintioctopunctata</i> | NIR Hyperspectral imaging | ASD FieldSpec Pro FR spectrometer | [89] |
| Tomato | <i>Rhizopus stolonifer</i> spores | NIR Hyperspectral imaging | Homemade built | [90] |
| Tomato | Leaves damaged by leaf miner | NIR Hyperspectral imaging | Nexus FT-NIR spectrometer | [91] |
| Tomato | Surface defects detection | NIR Hyperspectral imaging | ImSpector V10 | [92] |
| Tomato | Ripeness | NIR Hyperspectral imaging | ImSpector V9 | [93] |
| Leafy vegetables | Chlorophyll content | NIR Hyperspectral imaging | ASD Fieldspec FR spectroradiometer | [78] |
| Spinach | Quality during storage | NIR Hyperspectral imaging | EMCCD Luca-R camera-Hyperspec [®] VNIR | [94] |
| Spinach | Crop canopy under water | NIR Hyperspectral imaging | Specim V10 spectrometer | [42] |
| Watermelon | Lycopene, -Carotene, and Total Soluble Solids | NIR Hyperspectral imaging | NIR On-Line [®] X-One | [95] |
| Lettuce | Plant traits under extreme temperature and salinity stress treatments | Hyperspectral and Fluorescence imaging | Series VNIR Micro-Hyperspec Sensor; Fluor Cam 800 MF | [96] |
| Tomato | Growing of truss | MRI, NMR | Homemade built | [97] |
| Tomato | Maturity | MRI | 1 T MR system | [98] |
| Cucumber | Internal freeze damage | MRI | 9.4 T MR system | [99] |
| Bean | Pod water content | NMR | Homemade built | [100] |
| Tomato | Qualitative | VIS/NIR | LabSpec 5000 | [101] |
| Tomato | Antioxidants | VIS/NIR | HandHeld 2 TM | [102] |
| Tomato | Lycopene and physicochemical parameters | VIS/NIR | Varian Cary 500 | [103] |
| Tomato | Varietal discrimination | VIS/NIR | USB2000 spectrometer | [104] |
| Tomato | Transgenic lines discrimination | VIS/NIR | FT-NIR spectrometer | [105] |
| Tomato | Harvest time | VIS/NIR | AgroSpec VIS-NIR spectrophotometer | [106] |
| Tomato | Drought stress | Chlorophyll fluorescence | Handy FluorCam FC 1000-H system | [107] |

Table 2. Cont.

| Plant Species | Traits | Type of Analysis ^a | Instrument | Reference |
|------------------|--|--|-------------------------------|-----------|
| Chicory | Cold stress | Chlorophyll fluorescence | CF Imager | [108,109] |
| Bean | <i>Pseudomonas syringae</i> infection | Chlorophyll fluorescence | Fluorcam | [110] |
| Bean | Botrytis infection, magnesium deficiency | Chlorophyll fluorescence and thermoimaging | Homemade built | [111] |
| Bean | Photosynthetic traits, morphological parameters and shoot architecture | PAM fluorimetry | Growscreen Fluoro | [100] |
| Chinese cabbage | Quality | PAM fluorimetry | FluorPenFP 100 fluorimeter | [112] |
| Melon | Grafting compatibility | PAM fluorimetry | Imaging-PAM fluorometer | [113] |
| Tomato | Fruit ripening | Chlorophyll and polyphenol content | Multiplex sensor | [114] |
| Leafy vegetables | quality in post-harvest storage | Chlorophyll content | SPAD-502; Agriexpert CCN 6000 | [115] |
| Pepper | Canopy and plant architecture | RGB | Homemade built | [116] |
| Tomato | Canopy | 3D imaging | Digital camera | [117] |
| Tomato, Eggplant | Fruit morphology and shape | Tomato analyzer | Scanner imaging | [118–120] |

^a NIR = Near Infrared; MRI = Magnetic Resonance Imaging; NMR = Nuclear Magnetic Resonance; VIS = visible spectrum; PAM = Pulse Amplitude Modulation; RGB = Red-Green-Blue; 3D = Tridimensional.

5.1. Hyperspectral Sensing Studies

In Solanaceae, several studies report the use of hyperspectral sensing devices for early detection of various symptoms related to biotic stresses. For example, in tomato and eggplant, damages in leaves due to “early blight” (*Alternaria solani*) and the 28-spotted ladybird (*Epilachna vigintioctopunctata*) were studied [89]. A handheld ASD FieldSpec Pro FR spectrometer (Analytical Spectral Devices, 2002; 350–2500 nm) measured both diseased/infested and healthy leaves, and a regression model was established to assess the predictive power of the relationship between disease incidence and differential reflectance between samples. For *A. solani*, the red-edge subregion (690–720 nm) was the best at discriminating chlorophyll-related stress, due to chlorosis mediated by pathogens samples. For *E. vigintioctopunctata*, the highest differences were in the near-infrared region (720–1300 nm), due to loss of foliage area and density as well changes in canopy characteristics [89]. Diagnosis of *Rhizopus stolonifer*, a fungal disease causing postharvest losses in tomato, was done by means of a homemade system based on a combination of radiometry and spectrophotometry [90]. The model applied allowed to define spectral difference between inoculated and non-inoculated fruits in the 600–700 nm range. Leaf reflectance spectra have been also been adopted in tomato for diagnosis of leaves damaged by leaf miner, using a Nexus FT-NIR spectrometer (Thermo Nicolet Corporation, Madison, WI, USA; 800–2500 nm) equipped with a bifurcated fiberoptic probe, an interferometer, an InGaAs detector, and a wideband light source [91]. The results showed the occurrence of reflectance values of 800–1000 nm in damaged leaves due to degradation of cell components caused by the miner, and with increased severity of damage, values near 1450 and 1900 nm were observed, due to the decrease of water content.

In addition to phytopathological diagnosis, hyperspectral imaging was reported for early detection of qualitative defects to determine differences in ripeness stages in tomato. For the former objective, analysis of surface bruises due to blossom-end rot, sunscald, and cracks (which, even if not visible, represent the starting point of various diseases deteriorating the quality in pre- and postharvest) was carried out. A system combining an ImSpector V10 spectrograph (400 and 1000 nm) with a monochrome camera and zoom lens for detection of nonvisible and visible wavelengths, respectively, was used [92]. Data analysis indicated the wavelength region between 640 and 750 nm appropriate for bruise detection. For the second objective, spectral images (recorded using 1000 W

Tungsten halogen light as light source, a Hitachi B/W camera with an ImSpector V9 and a NIKON 55 mm lens) were compared to color RGB images [93]. Results indicated how spectral imaging is more suitable for classifying ripeness stage, reducing the error due to small differences in ripeness. These studies demonstrate the utility of hyperspectral devices to determine plant diseases and parameters related to quality, thus enabling improvement of the use of chemicals in horticulture in the various stages of the supply chain. Furthermore, the development of predictive models is essential to determine the appropriate wavelengths able to estimate accurately the phenotype of interest.

Hyperspectral reflectance has been used to investigate chlorophyll content in leafy vegetable varieties with different leaf colors (from yellow to dark) and different N treatments [78]. Reflectance spectra were obtained using an ASD Fieldspec FR spectroradiometer (Analytical Spectral Devices, Boulder, CO, USA) with a wavelength range of 350–2500 nm, and data was processed with the Unscrambler 9.5 software (CAMO PROCESS AS., Oslo, Norway, 2005). Spectrophotometry was also used to assess chlorophyll concentration. The spectral indices showed a good correlation with chlorophyll content, suggesting that NIR measurements hold promise for the assessment of chlorophyll content at the leaf level for green-leafy vegetables.

Application of hyperspectral images was also reported for the supervision of quality deterioration during different storage conditions in ready-to-use leafy spinach (*Spinacia oleracea*) [94]. The system consisted of a push-broom EMCCD Luca-R camera (Andor™ Technology, Belfast, Northern Ireland) equipped with a spectrograph Hyperspec® VNIR with a spectral range of 400–1000 nm and combined with a platform (MoCo DC Motor Controller, Micos, Auburn, MA, USA) that moved under the camera and in which leaves were positioned. The acquisition and storage of the images were made through a specific software (Headwall Hyperespec®, Headwall Photonics Inc., Bolton, MA, USA). The authors implemented different multivariate models for data analysis to observe how the deterioration of quality is related to the destruction of the internal structure of tissues, which was found to cause a decrease of the refractive index within the leaves and of the reflectance of the spectra. These two phenomena are related to the increase of free water in the tissue and the penetration of light [94]. The hyperspectral images analysis system represented a clear advantage compared to the spectrophotometric methods that analyze a small area of leaf sample, since it allowed for the identification of leaf regions with different states of degradation and the establishment of decision rules about the overall quality. Another employment in spinach has regarded the use of NIR to estimate crop variables in the greenhouse under combined water and nitrogen stress [42].

Quantification of bioactive compounds and chemical traits by means of NIR was reported in watermelon (*Citrullus lanatus*) [95]. The authors collected reflectance spectra in a high carotenoid content variety, cultivated over different seasons, by using a NIR On-Line® X-One (Buchi, Flawil, Switzerland; 900–1700 nm), equipped with a diode array detector (DAD) and a tungsten–halogen dual lamp. The application of a multivariate model evidenced wavelengths able to predict the content of phytochemicals in fruits, with a margin error below the 20%, demonstrating how the method can be applied for routine on-site assessment.

Combination of hyperspectral and fluorescence imaging was reported for lettuce (*Lactuca sativa*) to test phenotypic changes in early stages of development in plants subjected to extreme temperature and salinity stress treatments [96]. By means of an A-Series VNIR Micro-Hyperspec Sensor (Headwall Photonics, Fitchburg, MA, USA; 380–1012 nm), changes in chlorophyll and anthocyanin contents were measured, while a Fluor Cam 800 MF (Photon Systems Instruments, Brno, Czech Republic) was used to measure the photoinhibition due to abiotic stresses, leaf area, and growth rate. Results evidenced how lettuce has a reduction in plant growth caused by extreme salinity, while no growth rate decrease was evidenced under temperature stresses. Moreover, no permanent damages to the photosynthetic apparatus was registered due to high and low temperatures, demonstrating the recovery of plants after readjustment to the optimal temperature. The potential role of hyperspectral imaging in selection of cultivars was evidenced, given the strong relationship between green- and red-leaf color intensity and chlorophyll and anthocyanin content, respectively.

5.2. Magnetic Resonance Studies

Various applications of magnetic resonance (MR) for assessing crop physiological status and quality of production and screening for biotic diseases have been recognized. MR imaging (MRI) systems rely on the quantitative measure of the presence and the movement of water in plants, for which organs compete, in particular during fruit development. One of the major constraints for the measurement of sap-flow (the fluid transported in xylem cells), is the extreme sensitivity to invasive experimentation [121]. MRI and NMR are able to overcome this gap, since they record the displacement of water inside the plant through imaging analysis. Examples have been reported in vegetables, where researches assessed physiological changes in plants during growth and in fruits in pre and postharvest.

In tomato, xylem and phloem translocation and effects on growing of truss have been measured using a homemade built MRI system consisting of electromagnet with two gradient plates and a 50 mm air gap providing free access for the plant to the center of the magnet. The functioning of the system and the induction and detection of the NMR signal is fully described by [97]. The plant was left growing in the imager and, for 8 weeks in which long-distance transport into the truss was monitored, it was revealed that at least 75% of the net influx into the fruit occurred through the external xylem and about 25% via the perimedullary region (phloem and xylem). The approach indicated how the flux continued after the fruits and pedicels were removed, suggesting they are not involved in generating or conducting the circulation of sap-flow. Moreover, the system allowed the estimation of a loss of about one-half of net influx due to evaporation.

The potentiality for using MRI for fruit maturity classification is also reported in tomato [98]. The authors investigated changes in fruits spanning six stages of maturity (from green to red) with a 1 T permanent magnet MRI system (Aspect AI, Industrial Area Hevel Modi'in, Shoham, Israel). Imaging results and data analysis evidenced the ability of MRI to detect structural and physiological changes in fruits at different ripening stages, providing a valid method for predicting tomato maturity.

MRI was also used for cucumber (*Cucumis sativus*) to assess internal freeze damage in pickling fruits [99]. The experiment was conducted using a 9.4 T (400 MHz for protons), 89 mm vertical bore MR system (Varian, Inc., Walnut Creek, CA, USA) equipped with triple axis gradients (1000 mT/m) and a 4 cm Millipede[®] transmit/receive radiofrequency coil. Results demonstrated that MRI is a successful tool allowing determination of distinct regions related to the damages after just 150 min of exposure to freeze.

Application of NMR was reported in bean, where it was used to determine pod water content during growth [100]. A 1-week-old pod was inserted into an NMR portable service (described in [122]) and grown for 2 weeks, during which dynamic changes in the plant water status were assessed. Throughout this period, the bean pod water content was strongly affected by the light–dark cycle, and the growth rate was greater during the night. At the end of the period of the experiment, the pod began to lose water during the day and exhibited growth rates close to zero at night, although the dry matter was still increasing. The experiment allowed determination of water content during the whole growth period, and the pod remained apoplastically connected to the rest of the plant having the same changes of the water potential as the vegetative part of the plant.

5.3. VIS/NIR Studies

Several studies have been reported in tomato, using VIS/NIR for the determination of qualitative parameters, varietal discrimination, and harvest time. Color main descriptors, soluble solid, water content, pH, and firmness were assessed by means of a field spectrometer (LabSpec 5000, Analytical Spectral, 350–2500 nm) in the model species “MicroTom” grown in controlled conditions [101]. Reflectance spectra have been combined with destructive in vivo measurements, developing an ad hoc predictive model able to link wavelengths with the parameters of interest. The authors reported the related cross-validation error to be less than 6% for chemical and color traits and about 10% for water content and firmness, thereby demonstrating the effectiveness of VIS/NIR for high-throughput assessment of biochemical changes during tomato fruit development and ripening. VIS/NIR has

also been applied for the determination of physicochemical parameters—lycopene and polyphenol content—in diverse tomato cultivars and hybrids cultivated in two studies by means of FieldSpec HandHeld 2™ (Analytical Spectral Devices Inc., Longmont, CO, USA, 325–1075 nm) [102] and a Varian Cary 500 UV–vis–NIR scanning spectrophotometer (Varian Inc., Palo Alto, CA, USA) equipped with an integration sphere (Labsphere Inc., North Sutton, NH, USA) [103]. In both studies, the authors reported that bioactive compounds were accurately predicted with a cross-validation error below the 10% between reflectance spectra and lab measurements, while for the other physicochemical traits (i.e., brix, firmness, pH, titratable acidity), prediction was less accurate, partly due to a low variability of these parameters among samples.

The ability of VIS/NIR to discriminate plant genotypes has been investigated. An approach carried out on two tomato varieties cultivated in a controlled environment and assessed by a USB2000 spectrometer equipped with a grating, a detector (350–1100 nm, resolution 1–5 nm), and an optical fiber cable is reported in [104]. The discriminant analysis performed on various measurements in different top canopy leaves allowed development of a predictive model able to separate the two cultivars with accuracy up to 93% [104]. In a second study, the discrimination of nontransgenic and transgenic tomato lines, antisensed for the ethylene receptor genes *LETR1*, was carried out using an FT-NIR spectrometer system (Thermo Electron Corp., Madison, WI, USA) fitted with an optic fiber cable and a 50 W quartz–halogen light source [105]. Variations in the raw spectra—due to the diverse biochemical composition of leaves as a result of the transgene—were recorded. A derived regression model was able to discriminate with an accuracy up to 100%.

The feasibility of VIS/NIR to determine the best harvesting time in tomato has been also investigated [106]. By means of a mobile fiber-type AgroSpec VIS–NIR spectrophotometer (Tec5, Oberursel, Germany; 350–2200 nm), three cultivars were assessed at regular intervals, from green to fully ripe, and the predictive model has allowed to develop a new index measuring the growing stages of tomato based on the ratio of the growing to the on-vine duration before harvesting. The authors observed how VIS/NIR, combined with appropriate models, can be successfully adopted for predicting harvest time of tomatoes, allowing the implementation of autonomous remote sensing techniques such as fruit-picking robots.

The potentiality and the application of NIR to different fields of study are therefore innumerable, and the development of an appropriate predictive model is the key to success for phenotyping. However, it must be taken into account that several variables can affect the model (open-field measurements, irrigation treatments, diversity of raw materials) demonstrating how, particularly for qualitative assessments, the application of these sensing devices are better performing in a controlled environment in the presence of high uniformity [102].

5.4. Chlorophyll Fluorescence and Content Studies

Chlorophyll concentration is one of the targets pursued in phenotyping, as it is highly informative on the photosynthetic potential and of the health status of plants and, indirectly, of the nutrient content and nutritional value of production [123]. The analysis of chlorophyll fluorescence (CF) is based on the measurement of flux of light energy converted by the photosynthetic apparatus (blue light to red light). A low conversion indicates reduced photosynthetic assimilation as a consequence of a stress [124]. The magnitude of the yield of CF is an efficient method for screening and early detection of both biotic and abiotic stresses in plants [125]. PAM fluorometry is a method providing a fast assessment of the overall photosynthetic activity of the crop and its spatiotemporal variations through comparison of all relevant chlorophyll fluorescence parameters. PAM operates on a series of signal pulses by measuring the increases and decay of the light-harvesting antennae of the thylakoid membranes [124]. In tomato (*Solanum lycopersicum*), CF imaging (CFI) has been used to investigate drought tolerance in transgenic plants carrying the homeodomain-leucine zipper (HD-Zip) transcription factor ATHB-7, which acts as a growth regulator in response to water deficit in *Arabidopsis* [107]. Leaves of plants grown under well-watered and drought conditions were analyzed using the Handy FluorCam FC 1000-H system

(Photon Systems Instruments Ltd., Brno, Czech Republic). Before each measurement, which occurred in different cycles of stress and post-rehydration periods (2 days), plants were adapted to dark after placing the leaves in an aluminium foil leaf holder and then exposed to actinic light. The results reported the enhanced resistance to drought stress of transgenic plants when compared to the wild type, confirming the potentiality of the fluorescence-based screening technique for routine assay of plant's health performance and quantification of drought tolerance in tomato.

Cold stress tolerance evaluations by means of CFI were reported in chicory (*Cichorium intybus* L.) [108,109]. In these studies, measurement of the induction and relaxation kinetics of the chlorophyll fluorescence in plants cultivated at different combinations of light intensities and temperatures were made through an imaging fluorescence system (CFImager, Technologica Ltd., Colchester, UK). The results allowed detection of those parameters important for evaluating the effect of stress in terms of severity, affected processes, and acclimation to lower growth temperature. The authors demonstrated how young industrial chicory plants can be adapted to cold temperature as well as how the approach is efficient for abiotic stress screening.

CFI for biotic stress detection was also reported in bean (*Phaseolus vulgaris* L.) for the analysis of the response elicited after inoculation with *Pseudomonas syringae* pv. *phaseolicola* and pv. *tomato*, which mediate the compatible and incompatible interaction, respectively [110]. A FluorCam (Photon Systems Instruments, Brno, Czech Republic) was adopted to measure red chlorophyll fluorescence in infected plants, identifying those parameters able to detect them before the appearance of visual symptoms and maximizing the differences between the compatible and the incompatible interaction.

The combination of chlorophyll fluorescence imaging with thermal infrared provided a discriminating method for presymptomatic visualization of injuries and infections of *Botrytis* as well magnesium deficiency in young leaves of bean [111]. A robotized system using three cameras positioned sequentially above the plant leaves and able to move on three axes (XYZ) was programmed to acquire thermal, fluorescence, and video images on a surface of 50 × 50 mm at regular intervals (30 min) and analyze the stored images by means of an appropriate software [126]. The detection of symptoms were already possible within 21 h after the application of the fungus. The thermographic images were able to detect differences in temperatures over the leaf surface due to stomatal closure and low evaporation rate upon infection. Fluorescence, on the other hand, showed brighter zones due to the lower conversion of light energy by the photosynthetic apparatus, causing a higher fluorescence emission level. Both phenomena were related to the effects of infection on transpiration and photosynthesis. Chlorophyll fluorescence imaging proved effective also in the presymptomatic detection of leaf chlorosis associated with nutrient deficiency, while thermography detected necrosis-associated symptoms. The potentiality in the early detection of stresses of the methodologies were considerable and better fit with a protected environment, where weather conditions and solar irradiation—two factors hampering thermography and fluorescence imaging—can be controlled.

In bean, PAM fluorometry was used for screening of photosynthetic traits through the computerized high-throughput non-invasive screening fluorescence imager (Growscreen Fluoro) [100]. The system—which allowed completion of up to 60 plants per hour—was able to perform automated data analyses and extract morphological parameters and shoot architecture from the chlorophyll fluorescence images. The platform has been demonstrated to fit well to functional genomics studies aimed to reveal influences of mutations, transgenes, or environmental factors on diverse phenotypic properties of the plants, helping to select those with enhanced resource use efficiency or stress tolerance.

In Chinese cabbage (*Brassica chinensis* L.), PAM fluorimetry (FluorPenFP 100 fluorimeter; Photon System Instruments, Drasov, Czech Republic) has been used in a multiapproach study aimed to measure the decline in fresh and dry mass accumulation by comparing combined narrowband red and blue light-emitting diodes to broad-spectrum high-pressure sodium lamps (HPSL) [112]. The authors demonstrated how, under all conditions studied (plant ages and light intensities), the average chloroplast photochemical efficiency was slightly lower with the light emission spectra

obtained with LEDs, concluding that LED-grown plants have lower total mass accumulation, smaller leaf area, and minor sugar accumulation.

PAM fluorimetry was also reported in melon to study grafting affinity by measuring stress related to the change in chlorophyll fluorescence in grafted and ungrafted plants [113]. By means of an imaging-PAM fluorometer (Walz, Effeltrich, Germany), parameters highly sensitive to the stress and able to identify changes in plant performance after grafting were selected. The study demonstrated how PAM can be successful in evaluating highly compatible scion/rootstock combinations.

In addition to the above-described studies, it is possible to evaluate the chlorophyll content to determine fruit ripening and the overall quality in crops. Examples are reported in tomato and leafy vegetables. In tomato [114], by means of a handheld multiparametric sensor (Multiplex® 3, Force-A, Orsay, France), different indices were determined, reflecting the changes in the composition and distribution of pigments in pre- and postharvesting due to physiological changes during maturation. Comparison with other reflectance and remittance methods was made, evidencing how fluorescence enabled more precise differentiation and a superior quality.

In various leafy vegetables, an approach aimed to determine the quality in postharvest storage has been carried out evaluating the chlorophyll content through the measurement of the absorbance at certain wavelengths in leaf tissues [115]. Two meters, chlorophyll (SPAD-502, Konica Minolta, Chiyoda, Japan) and nitrogen (Agriexpert CCN 6000, Satake, Hiroshima, Japan), able to produce data within short time after sample collection, were used. The two methods were reliable for different species with different leaf-green color and morphology, with the exception of cabbage due to its unmeasurable thick leaf and pale color. In vivo data were then compared to high performance liquid chromatography (HPLC) for chlorophyll a and b measurement in leaves. Both measurements showed a high correlation and gave comparable grouping of vegetables with high and low content of chlorophyll, revealing the potency of in vivo observation for the prediction of freshness and nutritional quality in commercial leafy vegetables.

5.5. RGB and LIDAR for Morphology and Canopy Assessment

Plant and fruit morphology are other important objectives in phenotyping. Efforts in vegetables involved the use of RGB and CIR cameras and imaging scanners combined with appropriate software and related algorithms for image analysis and morphology parameters detection. A database comprising more than 150 analysis programs for different types of measurements in various target organs is available at the link <http://www.plant-image-analysis.org/> [127].

Canopy and plant architecture studies were conducted in an intraspecific, recombinant, inbred population of pepper (*C. annuum*), using RGB cameras and 3D imaging [116]. The system used was developed in the framework of SPICY Project (Smart tools for Prediction and Improvement of Crop Yield KBBE EU-FP7 program, <http://www.spicy-project.eu/about/about-the-project/>) and consisted of recording devices mounted on pipes distanced 60 cm from each other and positioned on a manually moveable trolley with wheels used as the basis. Plants were positioned in a glasshouse in four compartments of $12 \times 12 \text{ m}^2$ at a density of 6.8 plants/ m^{-2} . Since the distance from the camera to the plants was very short, to cover the whole height of plants (about 3 m), high resolution cameras with a very large field-of-view lens were used at four height levels, 75 cm apart. Two types of cameras were used: (1) RGB color camera with a resolution of 1024×1280 pixels and wide angle lens with a 75° field of view; (2) low resolution range cameras using radio-frequency modulated light source with phase shift detectors with a resolution of 48×64 pixels and 40° of field of view. Moreover, a system aimed to have a large field of illumination (to avoid any disturbance from ambient lights) was used. All images were recorded and stored on a hard disk for offline data processing. Details on the manufacture of instruments are reported in [116]. Appropriate algorithms were used to reconstruct 3D information from digital images. The approach was capable of assessing leaves' features (size, angle, area) and plant height, allowing identification-related QTLs. The challenges of these phenotyping

systems require overcoming the differences in light in greenhouses and acquiring the ability to observe plants from all directions by converting 2D-RGB images to 3D images.

In tomato, LAI has been estimated by taking digital photographs (2592×1944 pixels) by using a digital camera (Olympus C5060) and reconstructing the 3D CAD model by means of the photogrammetric package PhotoModeler Pro 5 (Eos System Inc., Vancouver, BC, Canada) [117]. The system allowed measuring approximately 400 3D points, belonging to the canopy surface, to assess with high accuracy the exterior surface of tomato plants inside of a greenhouse for the calculation of canopy volume. The proposed system, although effectively applicable in open field, appears to not be viable in terms of costs in such conditions, though it can more efficiently be applied for measurements in controlled environments.

Fruit morphology parameters have been assessed in eggplant and tomato by means of scan images and use of the Tomato Analyzer software (TA) [128]. This application has allowed high-throughput measurements of internal and external traits from scanned images of longitudinally or transversely cut fruits. Moreover, color measurements are possible by using color standards during the acquisition of images. The software has been firstly used in tomato to identify QTLs in three F2 populations constructed from crosses between elongated *S. lycopersicum* cultivars and the wild species with small fruits *S. pimpinellifolium* [118]. The researchers were able to detect novel QTLs related to fruit morphology and confirmed previously identified QTLs for fruit weight. For the latter, the approach was further helpful in finding those fruit parameters that are mainly involved in the variation of weight. In tomato and eggplant, TA has been used for characterization and discrimination of local varieties in combination with conventional methods [119,120], thus demonstrating itself to be a powerful tool for studying the diversity of germplasm, for the typification and classification of local varieties, and for the selection and breeding of novel cultivars.

6. Conclusions and Perspectives

In the age of “omics” sciences, where different disciplines are integrated to elucidate various biological processes, the objective of modern plant phenotyping is to deliver, with high accuracy and throughput, a large amount of quantitative data. To achieve this target, progress in automation, sensing technologies, software, and databases have been rapidly reached in the past years. Although field crops and model species paved the way of many advanced phenomics studies, the number of investigations carried out in vegetables have recently rapidly increased. Digital imaging has driven the advancement, allowing investigation of various aspects of crops, from physiological processes to architecture. The technology has demonstrated that it provides good predictors for the nutritional and health status of plants.

In this review, a range of nondestructive imaging techniques and associated portable devices for the assessment of plant architecture, qualitative and physiological traits, and biotic and abiotic stress, have been widely reviewed, addressing the vegetable sector. These technologies aim to obtain specific wavelength-derived indices in controlled environments and/or open field experiments, while also being highly informative, easy to deploy, and cost affordable.

Thanks to the progress in engineering and automation, systems combining different imaging techniques for phenotyping in controlled environments and open fields are being developed. These platforms are playing an extremely significant role in the increase of the precision and quickness of parameter estimations, giving the opportunity to accelerate the development of high-yielding and stress-resistant plant varieties and to identify genetic loci with high accuracy. However, it needs to be considered that, with fully automated high-throughput systems, the risk of data quality deterioration may occur if supervising at various stages is not implemented [4]. Therefore, for semiautomated tools, human intervention in processing and data checking is highly recommended during the various phases of the phenotyping process and data collection [4]. Despite the major advances in the field of phenomics, there are still drawbacks related to the accurate analysis of data. Unlike genotypes, phenotypes are impacted by the environment, raising the difficulty of associating environmental

metadata with phenomics data points. Therefore, successful use necessitates robust computational skills in the field of biostatistics with the ability to handle a large amount of data, develop mathematical algorithms, and draw out predictive models. The management of the huge amount of generated “big data” from various phenotyping systems is the actual challenge for the scientific community to overcome—from both technical and social perspectives—in order to avoid the transition toward a new bottleneck. In the former case, coordination efforts across research groups, development of appropriate analytical frameworks for processing and integration with other omics data, and cloud platforms for data storing are required. For the latter, ownership and privacy issues need to be addressed through policies to avoid the concentration of data in the hands of big agribusinesses, moving toward an increase in sharing and transfer of know-how [129].

Besides applications in breeding and omics studies, and notwithstanding the difficulties highlighted before, the development of continuous, precise, and fast phenotyping technologies in protected horticulture (including vegetable as well as ornamental plants) will have an impact on the evolution of precision crop management approaches, with positive effects on economic and environmental sustainability. The information derived by such monitoring of crop growth alongside environmental parameters will stimulate the progression of new greenhouses typologies and technologies for more efficient resource management. Furthermore, the automation of ever more crop management operations will consequently increase. It must be highlighted, however, that a parallel advancement of mechatronics in protected agro-environments is necessary to allow efficient collection and use of digital data acquired by the various sensing technologies discussed above.

It is evident that the development and implementation of all such novel technologies and approaches require a close multidisciplinary effort among users, including plant and crop physiologists, geneticists, breeders, engineers, modelers, ICT specialists, and others depending on the specific application.

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