



LemnaTec High-Content Screening

POPLAR PHENOTYPE ASSESSMENT

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Automated High-content Screening of Complete Plants

The LemnaTec Scanalyzer 3-D system combines comprehensive quantification of growth and phenotyping with high reproducibility and throughput, allowing long-term data storage for later data mining.

The large number of different parameters quantified automatically makes comprehensive high-content screening and statistics on morphological patterns and changes of patterns in time possible.

High-content screening is the next step beyond high-throughput screening: The term originates from cell screening, where in earlier times high-throughput screening assays were designed to produce a single relevant screening parameter, e. g. a staining or a change in absorption/fluorescence. In contrast to this, high-content screening produces a comprehensive set of data, related to one sample, in many cases based on image processing. In fact this is what many screening systems for complete plants always do, even if assessment is done by hand. For this progressive reason, screening programs with complete plants frequently focus on the high-throughput method, as high content is often already a reality. Nevertheless, combination with modern imaging and plant handling technologies can offer even larger numbers of parameters feeding the "content pipeline" of screening programs.

The LemnaTec Scanalyzer 3-D systems now combine the speed and automation of high throughput with the assessment of multiple parameters for each plant at each respective measurement point. Relevant data is then derived either from each single image (sizes, areas, colours, morphology), or calculated from changes in time in relation to growth or specific stress conditions. The final results are always specific phenotypic patterns for different plants or groups of plants, to be related to particular genes or used as basis for selection processes.

While the relevance of LemnaTec image processing for growth and abiotic stress reaction assessment is discussed extensively in various LemnaTec papers on Arabidopsis and corn, the following text focuses on measurement parameters for poplars, based on single images. These images were taken as pilot demonstration images to show the relevance of

different imaging parameters. As these images were not taken with a LemnaTec 3-D system, the focus here is on the demonstration of measurement parameters, and not on important features of high reproducibility and automated screening, like optimised imaging conditions (e. g. light, camera).

The following examples are parameterised and adapted for hybrid poplars, but similar approaches are available for a wide range of other biological applications.

Visible Leaf Area

LemnaTec generally quantifies visible leaf area from three sides: Two side images are made before and after a turn of 90° and a top image is made additionally. Due to growth structure and plant morphology, these images may have very different informational contents. While small plants usually contain more information on the top image, with increasing plant size the side image is gaining importance.

Visible leaf area is the two-dimensional projection of all visible leaves. Having this from three different directions provides comprehensive 3-D information on the visible leaf area.

Leaves not visible due to overlap are not quantified separately for two reasons:

1. Due to irregular shape and orientation in all three dimensions, a stringent extrapolation of partially covered leaves is not possible without use of uncertain hypotheses on their shape and position. This is particularly true if plants have a different genetic background, affecting the phenotype of the leaves and plant by (test)-design.
2. Covered leaves are part of the phenotype, thus e. g. reduction of solar use efficiency or reduced evaporation due to overlap are phenotypic features and compensating them may be misleading.

As shown below, changes in visible leaf area may be used for a wide range of biological assessments, e. g. drought tolerance tests.

Information Derived from Visible Leaf Area - Small Plants

The following pictures show the three images taken (side 1, side 2 at 90°, top) and the area-analysed colour-coded result for plant No. 1.



Side 1 original



Side 2 original



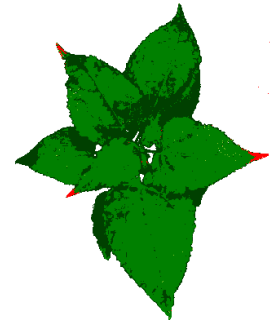
Top original



Side 1 colour classified area



Side 2 colour classified area



Top colour classified area

Original image and colour classified images of plant 1

Based on these three images, a leaf area or plant volume can be calculated, based on more or less plant-specific mathematical models. The most simple, but often quite effective one is the **image plant volume IPV**, defined as $IPV = \text{sqr}(\text{area side 1} \times \text{area side 2} \times \text{area top})$.

This allows, for example, absolute size comparison between plants and calculation of growth rates for each single plant as an often even more reliable parameter (please see separate LemnaTec paper on growth rates of Arabidopsis).

Additionally, based on this type of analysis **leaf areas from each single plant** can be compared for each perspective separately. To compensate for area differences between side images, it is recommended to take the average value of the side images for comparison between plants if only side images are to be compared.

The **ratio between top and side image area** may be a phenotypic trait, but can also be used very effectively for assessing, for example, fast dynamic processes like drought tolerance. As shown for a soybean plant of similar size as the poplars displayed here (see LemnaTec soybean application paper), leaf area ratio top/side varies extremely, allowing identification of drought symptoms and recovery. This may be easily used to induce specific watering regimes in LemnaTec conveyor systems with watering stations.

Also **leaf colours**, e. g. the distribution of different shades of green, can be compared. This allows e. g. advanced reproducible colour quantification on nutrient deficiencies . As seen on the pot image above, the LemnaTec system detects, for example, reddish leaf tips.

Plant height and maximum plant widths are also included.



Colour classified area image of plant 2

Morphological parameters describing compactness, symmetry and stockiness of plants may be analysed additionally, but here they did not show significant differences between the two plants and will be shown related to other examples below.

The following table shows a compilation of parameters measured for two plants:

| Parameter | Plant 1 | Plant 2 |
|-------------------------------------------|---------|---------|
| Height cm | 13.2 | 11.1 |
| Average width cm | 12.4 | 11.6 |
| Average side area cm ² | 275.2 | 216.3 |
| Top area cm ² | 202.4 | 126.7 |
| Average dark green side cm ² | 15.4 | 30.5 |
| Average medium green side cm ² | 125.0 | 115.4 |
| Average light green side cm ² | 134.4 | 69.3 |
| Average red side cm ² | 0.9 | 1.0 |
| Dark green top cm ² | 55.2 | 105.7 |
| Medium green top cm ² | 145.4 | 20.4 |
| Light green top cm ² | 0.3 | 0.3 |
| Red top cm ² | 1.2 | 0.3 |
| Image plant volume cm ³ | 3915 | 2434 |
| Top/average side ratio | 0.73 | 0.59 |

Larger Plants

For larger plants, relevant parameters to be measured are different, due to their specifically different shape.

Top images were not used for the following measurements, as the measured leaf area is more defined by deviations from vertical growth than by leaf width.

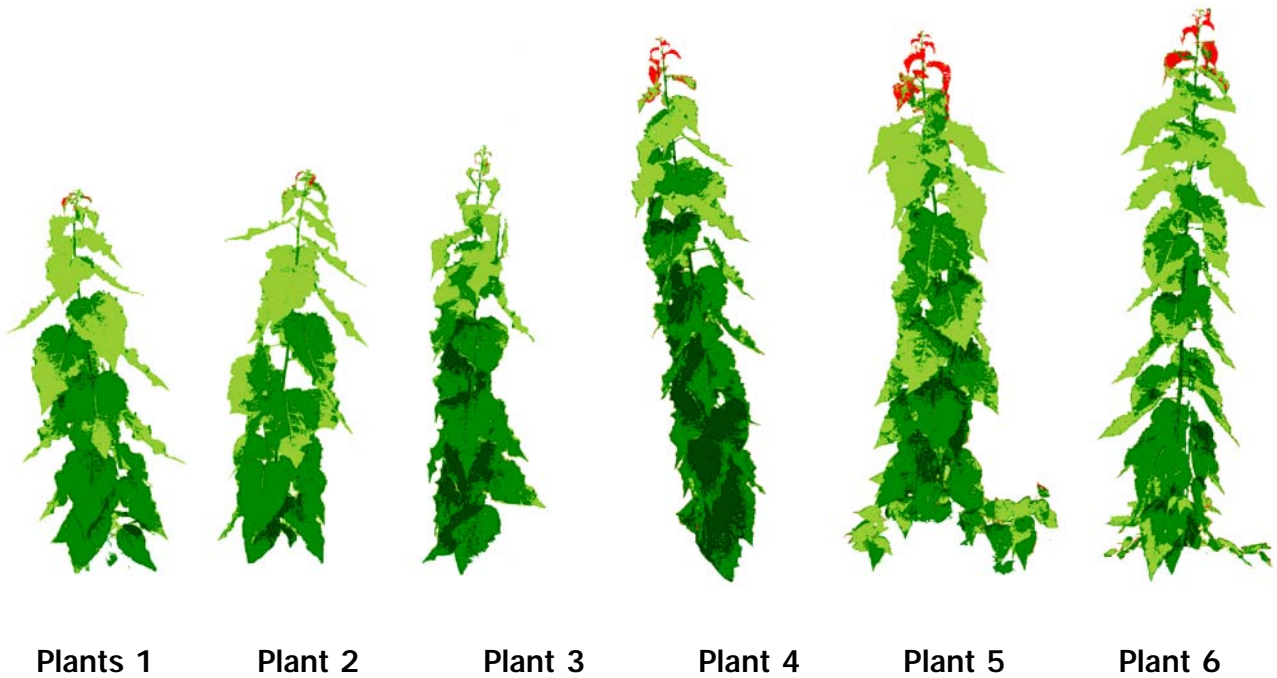
The plants 1 to 6 are put in order according to their increasing height.

Plants 3 to 6 show significant anthocyan formation in the top leaves, which is quantified in a red colour class.

The side branches of plant 5 and 6 are included in the analysis to demonstrate morphological parameters.

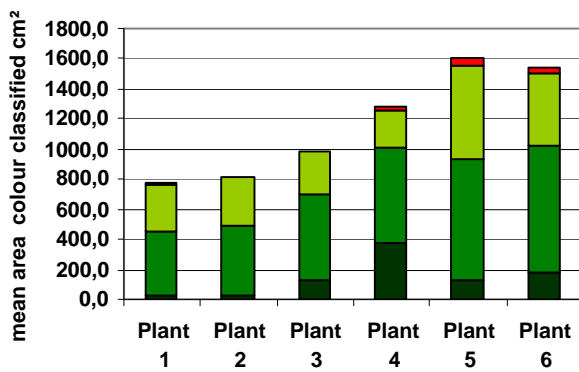
While only one side image per plant is shown below, all data represent average values of two side images, taken from a 90° angle. In some cases differences between these side images will be used as parameters for symmetry.

Each parameter presented in the table below will be discussed later.

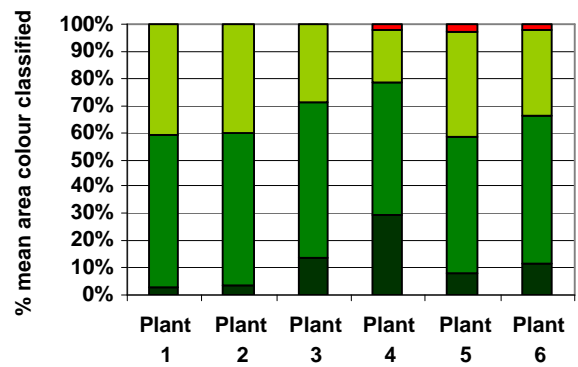


One of two side images taken from six poplar plants being analysed and colour classified

These two graphs show the average colour-classified area for all plants, in absolute values and in percentages of the total area of each plant:



Colour classified average areas for all six plants



Colour percentages for each plant

The leaf area quantification shows – as is also qualitatively obvious from the images – plant 6 being the tallest, but plant 5 having the highest leaf area, as leaves are much more compact in plant 5 and its side branches larger.

Additional Morphological Parameters

In addition to the area measurements above, the following table shows a set of morphological parameters for both plants. These parameters help to reproducibly classify the phenotype. This set of parameters just shows some options. Specific morphological traits may be developed from the images for particular biological traits.

.Morphological parameters for six plants as displayed above:

| | Plant 1 | Plant 2 | Plant 3 | Plant 4 | Plant 5 | Plant 6 |
|-------------------------------------------------------|---------|---------|---------|---------|---------|---------|
| height cm | 66,6 | 68,1 | 86,4 | 100,8 | 107,0 | 117,4 |
| max. width cm | 28,4 | 29,1 | 24,6 | 24,5 | 37,7 | 33,1 |
| mean area side cm² | 770,7 | 818,0 | 986,0 | 1282,5 | 1602,6 | 1542,9 |
| mean covered area width cm | 11,6 | 12,0 | 11,4 | 12,7 | 15,0 | 13,1 |
| image leaf volume (cylindrical) cm³ | 7003 | 7718 | 8834 | 12810 | 18839 | 15921 |
| circumferenceof leaf area/height | 6,3 | 6,2 | 4,4 | 4,5 | 5,5 | 6,2 |
| stockiness (roundness) | 230 | 220 | 150 | 165 | 222 | 342 |
| mean vertical moment | 0,029 | 0,028 | 0,021 | 0,015 | 0,025 | 0,020 |
| % difference vertical moment both side images | 3,7 | 1,6 | 7,5 | 5 | 38,1 | 12,7 |
| 2nd_moment_pricpal_axis_ratio | 12,1 | 12,3 | 20,4 | 32,6 | 19,4 | 29,3 |
| compactness sourround area % | 32,7 | 30,8 | 15,7 | 12,7 | 28 | 40,2 |

The parameter “maximum width” describes the maximum extension of the plant in horizontal direction. This means that sloping plants show higher values than strictly vertically growing ones. This way information on growth direction and plant width is provided.

The average covered area width describes how wide the leaf area would be if evenly and compactly distributed over the plant height. Thus a comparison between plants of different leaf density / compactness can be made.

The image leaf volume (cylindrical) is a first estimate of leaf volume, calculating a cylinder from plant height and side leaf area. As expected, plant 5 shows higher values than plant 6, as leaves are much more compact with plant 5.

The circumference of the whole leaf area divided by plant height is one option to estimate leaf density. For maximum density and a column shape the value would approximate 2.

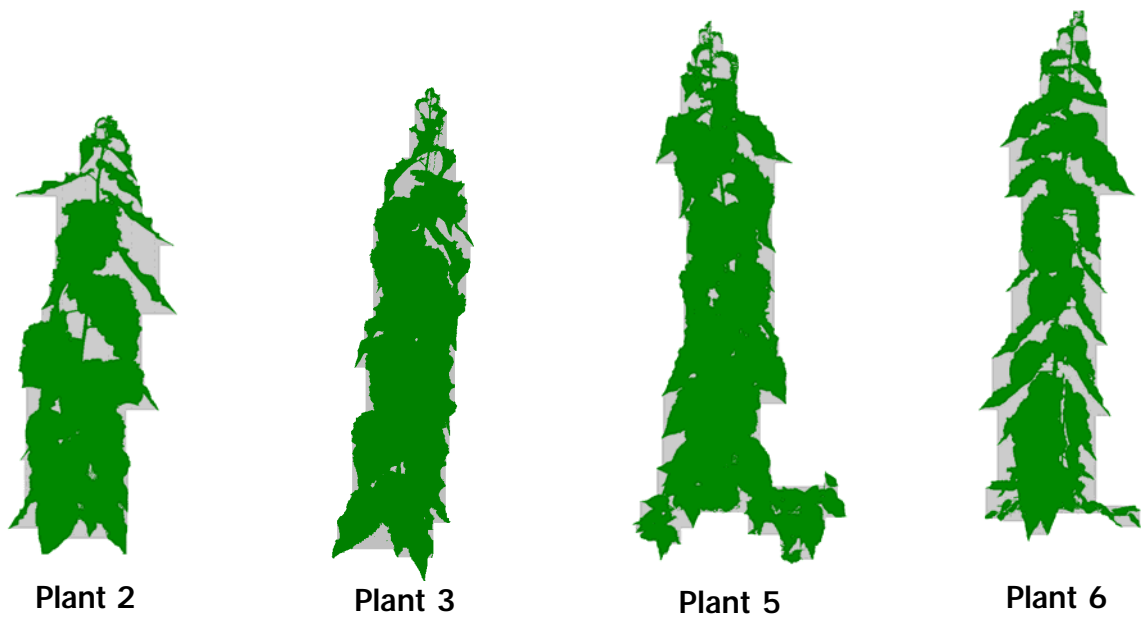
The lower the value, the more compact is the plant leaf coverage. Plant 3 and 4 are the most compact plants here. Stockiness is mathematically calculated as roundness. For objects being far from round, the term stockiness seems to be more appropriate.

The more compact a plant is, the lower are its average vertical moments. The rising values between plant 4, plant 5 and plant 6 are a result of their vertical branches. As these moments are calculated not strictly vertically, but following the main momentum axis (i. e. the main growth direction in this case), a decline of a plant has no influence on this value. Thus artefacts due to sloping plants are avoided.

The differences between the vertical moments of two side images provide a good estimation of symmetry as being visible from the top view, at the same time having eliminated any asymmetry caused by sloping plants. This is clearly shown by the 38 % difference of plant 5, caused by the side branches, compared to values below 8% for all plants without side branches (plant 1 to 4).

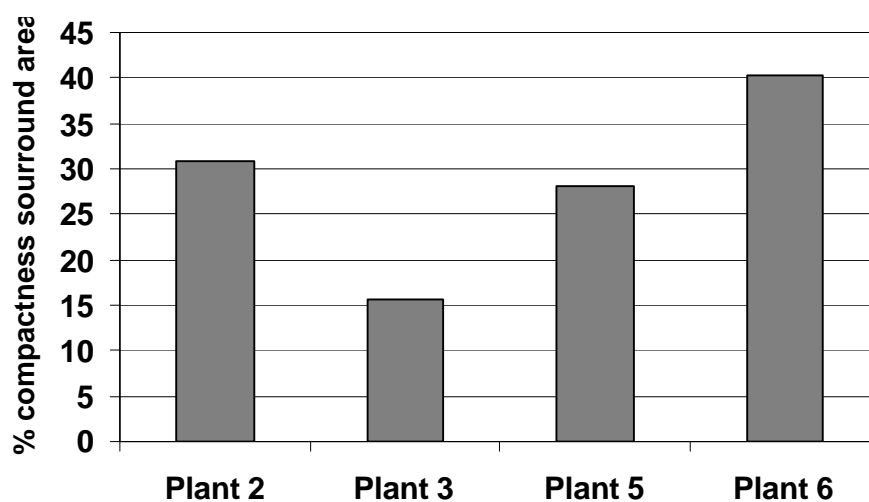
The second moment principal axis ratio defines the moment ratio of the two main axes. As poplars in general are growing higher combined with almost constant growth of width, this value tends to increase with size, as can be seen here, except for plant 5 due to the side branches.

While all these values were derived from the images shown above, the LemnaTec software offers additional options to extract further information from the image, as shown below.



Side projections for four poplar plants (green) with compactness surround area (grey)

To resolve the question of how compact or dense the leaves of a poplar are, the LemnaTec imaging software has been used to define surround areas in a user-defined range around each leaf, which could be attributed as “bays” or “holes” in an otherwise compact leaf structure.



Compactness surround areas for four plants as displayed above

This additional area is shown grey in the example images. While compact plants have almost no such areas, less compact plants have areas of up to 40% relative to their leaf area.

The examples for data extraction shown here may of course be customized.

Conclusions

1. LemnaTec can provide very powerful tools to assess a wide range of size-related and morphological parameters for plants. Different morphological parameters allow different, reproducible, easy to visualise groupings of plants. The paper provided here focuses on size-related parameters, to be used for growth rate measurements and additional morphological parameters of complete plants, like compactness, different moment values, stockiness etc.
2. Quantitative and reproducible assessment allows application of advanced grouping algorithms. Each quantitative parameter refers to a different morphological trait and may be used in high-content screening, e. g. for QTL-analysis or computational modelling of plants.
3. As all images – and thus a tremendous amount of raw data – are stored after a test, the LemnaTec image systems allow consistent reanalysis of ALL data over extensive and long screenings, even if some of the parameters to be assessed are not yet known at the beginning of the screening. This boosts the value of the screened plant library tremendously and also shortens screening times and reduces costs. Tests need not to be repeated because of additional assessments that just revealed themselves while screening was already on its way.
4. Routine use of control plants allows automatic and quantitative quality control on test conditions to maximise quality and reproducibility of results.

For further information please do not hesitate to contact LemnaTec

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