Quantification Method for In-Vitro Tissue Culture Plants Morphology using Object Tracking and Digital Image Analysis

Kestrilia Rega P[ab], Agus Buono[b], Sutoro[c], Irman Hermadi[b]

[a]Mathematics and Natural Sciences Departement, University of Surabaya, Jl. Raya Kalirungkut Tenggilis, Surabaya-East Java, Indonesia
[b]Computational Intelligence Research Lab, Dept. of Computer Science, Bogor Agriculture University, Dramaga Campus, Bogor-West Java, Indonesia
[c]BB-BIOGEN, Jl. Tentara Pelajar 3A, Bogor-West Java, Indonesia
kestrilia@ubaya.ac.id, pudesha@yahoo.co.id, wisnuj@cs.ui.ac.id, kusumo@cs.ui.ac.id

Abstract— **Manual measurement of morphology variables on in-vitro stored plants usually cause either physical damage or microorganism infection such that further monitoring of their in-vitro performance is precluded. This study adapted computer vision technology by which it is possible to conduct such measurement without physical contact or destructive test. Moreover, by applying object tracking and pattern recognition technique in the algorithm, the system could provide automatic and real time analysis. It was shown that this quantification method reach 80.2% and 87.9% in the measurement of leaf area and chlorophyll intensity. Intensity histogram and Fourier spectrum found to be the best feature for leaf recognition and interpolation usage to adjust pixel amount over the camera distance provide better estimation on leaf area.**

Index Terms— Image analysis, in-vitro, morphology, object tracking, pattern recognition, tissue culture

I. Introduction

Tissue culture is a technique for plant asexual propagation. It uses small pieces of plant tissue (explants) such as leaf, shoot or root which are cultured in growth medium composed of nutrient, sugar, vitamin and hormone under sterile condition (usually explants put into sealed bottle) [1]. Using the appropriate growing conditions for each explant type, explants can be induced to rapidly produce new shoots and roots then ultimately develop into a whole plant [2]. With this method plant propagation could be held without time, season or weather consideration. Moreover, in a relatively short period it could produce new plants all exactly alike.

For research purpose, quantification of plant morphology variables (e.g. leaf area, chlorophyll intensity, number of leaf, root and branch) is needed to apply data processing using particular statistical method for further analysis. But, it is known that manual measurement to provide such quantitative data usually cause either physical damage (destructive test) or microorganism infection such that future monitoring of their in-vitro performance is almost impossible. Therefore, some researcher [3,4,5] try to apply computer vision technique to avoid physical contact and destructive test. Measurement of the variables conduct on the plant's digital image and through image processing application, digital data were transformed into plant's morphology variables. Their experiments show good result, unfortunately the developed systems were not intended to provide automatic and real time analysis.

The goals of this project were to develop automatic and real time system by applying object tracking and pattern recognition methods on its algorithm. Considering the segmentation complexity because there was not any general segmentation procedure which can be used to identify leaf, branch and root all together. Leaf segmentation was chosen because of its high importance as growth indicator. Two morphology variables focused on this project were leaf area and chlorophyll intensity. Using this system, the tissue culture researcher could hold observation on plant morphology in simple and fast manner with detailed and accurate result.

II. Materials and Methods

Plant materiasl.

Manihot esculenta Crantz was chosen for this initial trial due to their high survival rate and good development during in-vitro culture procedure. Moreover, the culture media composition which is the key success for this plant growth was well studied in the laboratory where this project was conducted. Sixty explants were cultured in 8 centimeters height bottle using Murashige & Skoog medium, 3 of them died during the process, observation was set for 4 months in order to collect various plant's size image as the representation of its development stage.

Manual measurement.

This procedure was intended to provide actual measurement of the plant's morphology variables for system's evaluation and validation purpose. Each leaf mapped on the millimeter block paper, then all 1 mm^2

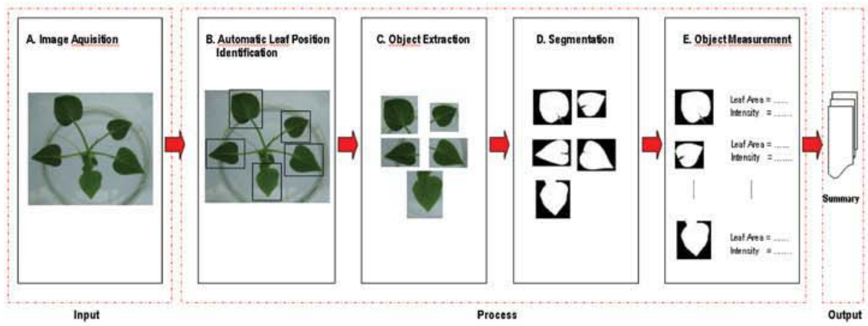

Fig 1. The global system's design

square within the leaf edge was counted as the actual measure of leaf area and chlorophyllmetre was used to produce actual measure on chlorophyll intensity.

Plans image.

Specification of digital camera used in the experiment was 7.1 MP effective pixels, 1/2.5 inch image sensor, 4.6 (W) - 17.3 (R) mm lens, 3.7 x optical zoom, 3 cm (normal) 60 cm (infinity) macro and 15-1/16000 sc shutter speed. Images of each plant were taken from 9 point of view, 1 from the top and 8 from the side of the bottle. Later, these images will be analysed to decide the best input for the system.

Computer hardware and software

To develop the system's prototype, MATLAB 6.5.1 was used in a notebook computer with 1.8 Ghz centrino processor, 715 MB RAM and 120 GB hard disk. MINITAB 14.0 and Microsoft Excel were used for statistical result analysis.

III. SYSTEM'S DESIGN

Figure 1 illustrated the global system's design. It was composed of 3 major parts which are plant's image acquisition as input; object tracking and object measurement algorithm in the process; and statistical summary as the output.

A. *Image Acquisition.*

The best image for system's input is plant's image taken from the top of the bottle. During the experiment which is conducted on 57 sets of plant images (1 set consist of 9 images), it was known that 66.2% of the total leaf could be seen from this point of view and only 49.41% from other point of view. Therefore, the plant's image which was taken from the top of the bottle should be used as system's input.

B. *Object Tracking Algorithm.*

Before execution of the algorithm, several window's sizes should be prepared to capture each leaf image from the plant whole image (input image). In order to get these sizes, K-Means Clustering method [6] was applied on 187 leaf images from 57 input images. It was then decided to construct 4 sizes of window ranging from the smallest one to capture small leaf images through the biggest one to capture bigger leaf images. The window sizes order are 41x58 pixels, 80x71 pixels, 109x104 pixels and 167x158 pixels. Here is the detail procedure of object tracking algorithm:

1. *Finding the position of each leaf in the input image.* First, the input image is processed with tresholding method to form its binary image. The treshold value set at 90. This value was the optimal one which was chosen by trial and error [7] among some other threshold value that divide leaf's pixels and background pixels. By analyzing some binary images produced by particular threshold value, it was known that values under 90 will cause many leaf's pixel disappeared and in the other hand values above 90 will cause many background pixels appear on the binary image. Second, labeling technique is applied on the binary image using 8-neigbours rule to identify all connected pixels. A group of connected pixels considered as one object, therefore identical label should be given to all of them. Then, each object's pixels amount and center coordinate stored in a particular matrix. The objects with pixel's amount less than 150 are ignored because it is obvious that such objects are not big enough to be considered as a leaf (the smallest leaf's pixels amount which is found among 187 leaf's image samples is 169). Third, image of each selected object is separated from the input image using cropping technique. The cropping window size (chosen from 4 window size available) determined by the pixels amount of corresponding object. For one input image there will be several sub images. Unfortunately, not all of these sub images really contain leaf image, hence the need of further selection procedure.
2. *Pattern matching to select correct sub images*. Correct sub images are those sub images which contain appropriate leaf image (image with complete leaf shape). For this purpose, 187 leaf images were used as training data. Each sub images being tested then compared with these training data. If the sub image's similarity with training data is high, it consider as correct sub image, otherwise it will ignored. Intensity histogram and Fourier spectrum [7] are the best feature for this selection among other feature already tried such

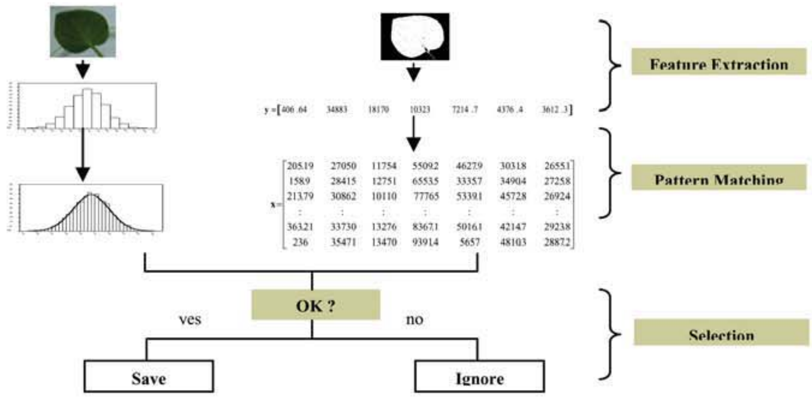

Fig 2. Ilustration of Pattern Matching on Sub Images Selection

as statistical texture and moment invariants. On average, both features could capture 85% of the total correct sub images it should be capture. To quantify the similarity, kullback discriminant measure (equation (1)) applied to intensity histogram matching and mahalanobis distance (equation (2)) applied to Fourier spectrum matching.

$$K(I,M) = \sum_{i=1}^{n} i_{ci} \cdot \log \frac{i_{ci}}{m_{ci}} \quad (1)$$

$$d(\mathbf{y},\mathbf{m}_x) = (\mathbf{y}-\mathbf{m}_x)^T \, \mathbf{C}_x^{-1} \, (\mathbf{y}-\mathbf{m}_x) \quad (2)$$

I and M are intensity histogram being compared, each of them have n-bins, i_{ci} and m_{ci} are the i-th bin frequency, y is a vector of sub image's feature value, x is a vector of training data's feature value, $\mathbf{m}_x$ is training data's mean vector and $\mathbf{C}_x$ is its covariance matrix. The smallest K and d, the highest the similarity. All sub images similarity measure then arranged in ascending order. Sub images with K above 0.3 and d above 24 were ignored and the rest of them then selected by voting. Therefore, all sub images which is remain after voting procedure is those selected by both feature. Figure 2 illustrated the pattern matching procedure.

C. Object Measurement Algorithm

1. *Correct sub images retrieval*, this procedure was done by accessing correct sub images database which is created during the object tracking algorithm.
2. *Segmentation* to each sub images by adaptive tresholding. This process will produce binary image for each sub image. In order to reconstruct leaf area, filling hole technique is applied to each binary image such that leaf area will no longer contain holes caused by improper tresholding.
3. *Object measurement*, consist of two measurement process which is leaf area and chlorophyll intensity estimation.

Leaf area estimation:
For each leaf, all object's pixels found in binary image were counted, then using equation (3) its total amount then converted to the leaf area estimation in mm^2.

$$a = 200 \cdot {}^{l}\!/_{f} \quad (3)$$

a is leaf area estimation in mm^2, l is total amount of object's pixels in binary image and f is total amount of conversion object's pixel. Conversion object in an object used to adjust pixel amount over the camera distance. It has 200 mm^2 of the actual area. Equation (4) is used to find f.

$$\ln(f) = 10.2574 - (0.405 \cdot x) \quad (4)$$

x is camera to leaf being measured distance, if $h=\ln(f)$, then:

$$f = e^h \quad (5)$$

This total pixel adjustment was applied because it was known that there was pixels amount reduction as camera distance increase. Moreover, it was proven that the model with such adjustment provide better estimation.

Chlorophyll intensity estimation:
First of all each sub image should convert to HSV from its RGB format, then v (value) component for each object's pixel withdrawn from $\mathbf{V}$ matrix. Chlorophyll intensity estimated using equation (6).

$$c = 50.9 - 54.4 \cdot \bar{v} \quad (6)$$

c is chlorophyll intensity estimation and $\bar{v}$ is the mean of object's pixels v component. Other image format were studied as well, and it was known that HIS and RGB format was not provide good estimation on chlorophyll intensity since their model was not good enough (justified by model's significance and its independent variable contribution to explain actual chlorophyll intensity variation) to describe the pattern of its components and actual chlorophyll intensity measure.

The object tracking and object measurement algorithm's flowchart is shown in Figure 3.

D. Application Program

The application program was developed to help user operate the system through its interface (Figure 4). This program was written in MATLAB programming language. Plant image is entered by the user and in no time the program will display each leaf area and chlorophyll intensity measurement followed by its descriptive statistics.

IV. RESULT AND DISCUSSION

The in-vitro tissue culture morphology quantification system would consider good if it could identify almost all leaf from a plant image and produce high accuracy on leaf area and chlorophyll intensity estimation.

A. Number of Correct Sub Images Actually found

Total number of correct sub images actually found was compared with those should be found by the system. On average, the system could found 88.12% of the total correct sub images. Minimum percentage is 25%. This lowest point happened when the system should work on difficult input image such as image of a plant with more than 10 leaf on it. In this condition, images characteristically have high numbers of overlapping leaf which cause each sub image very often contain more than one leaf with connected edge between them. This kind of sub image will be eliminated during pattern matching process because of its improper shape. The highest percentage point (100%) reach when the system worked on ideal input images which is those images with no overlapping leaf.

B. Actual Measure of Leaf Area and its Estimation Comparison

This actual measurement and system estimation comparison is described in Figure 5. It is shown that actual measurement and system estimation display very close pattern, indicate that the system could estimate the actual measure accurately. High difference found in some sample points which are 82, 115, 117, 136, 139, 143, 144 and 148. To quantify this comparison, Table 1 provides its descriptive statistics either in mm^2 or in percentage of the difference from its actual measure. In average, the difference of actual measure and system estimation is 23.41 mm^2, equal to 24.84% from the actual measure.

TABLE 1
DESCRIPTIVE STATISTICS OF THE ACTUAL AND ESTIMATE DIFFERENCE ON LEAF AREA

Statistics	Difference (mm^2)	Difference (%)
Mean	23.4100	24.840
Median	15.2000	19.710
St. Dev	24.4600	22.990
Variance	598.2800	528.440
Minimum	0.0780	0.108
Maksimum	153.9400	195.930
Range	153.8600	195.820

With this small enough value, the system performance said to be good. But, the standard deviation and variance indicate the presence of some extreme value lying far away from the mean point. This phenomena could clearly seen on the wide range of minimum and maximum value. In the histogram of the data, it was found 2 extreme values. Both values are over 100 mm^2 (the range of the actual leaf area is 15 – 189 mm^2). These values drags mean point to the right side (the side with high value) of the histogram. Therefore, the median was preferred rather than mean to describe system performance. The median value is 15.2 mm^2 equal to 19.71% from the actual measure. This value indicates that without the presence of those extreme values, the system's performance could be much better. Two of the factors cause extreme value are leaf's height measurement fault during destructive test and system's failure on sub image selection process where the system unfortunately select those sub images with overlapping leaf. This overlapping leaf will consider as one object since their edge connected one over another which

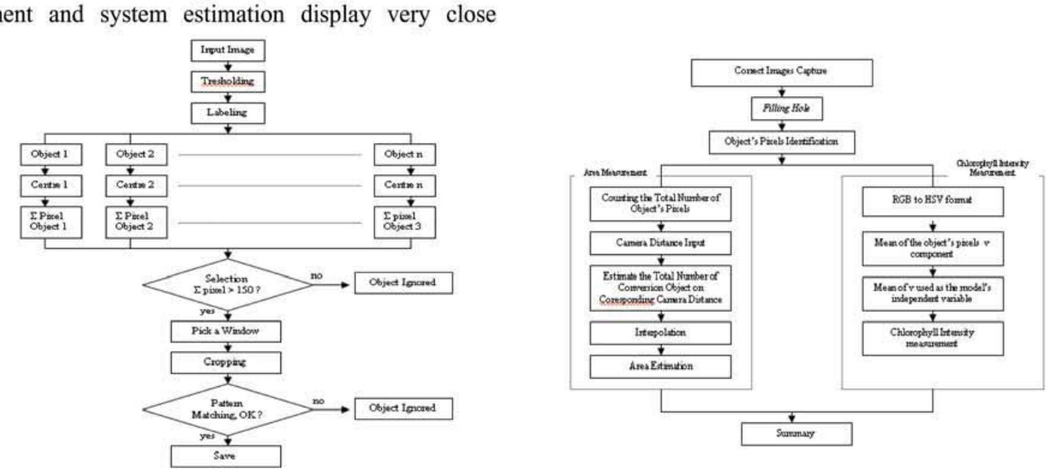

Fig 3. Flowchart of object tracking (left) and object measurement (right) algorithm

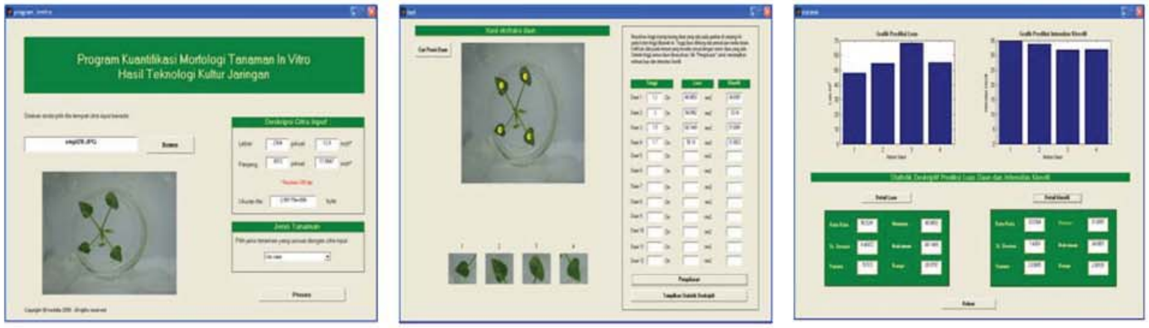

Fig 4. Interface of the application program

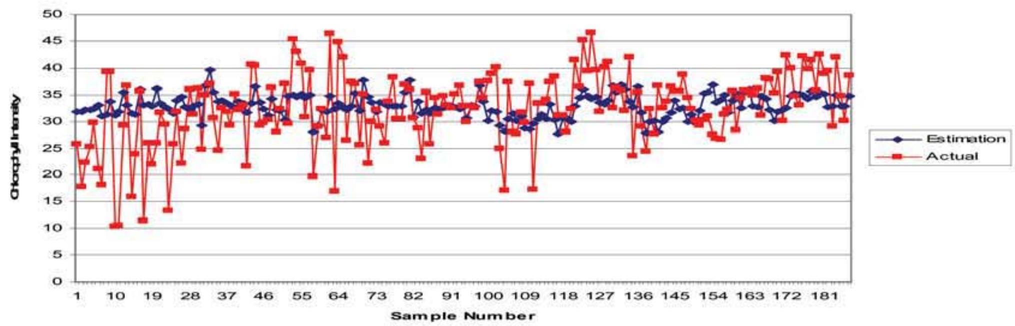

Fig 5. Line chart of actual measure v.s. system's estimation on leaf area

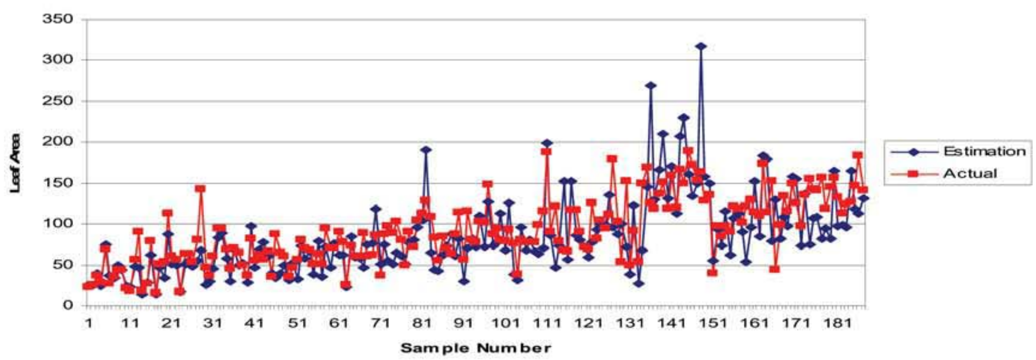

Fig 6. Line chart of actual measure v.s. system's estimation on Chlorophyll Intensity

cause higher estimation of the leaf area.

C. Actual Measure of Chlorophyll Intensity and its Estimation Comparison

The comparison of actual measure and system estimation on chlorophyll intensity is described in Figure 6. It is shown that their average value overlap in the same line. But, the actual value seems to be more fluctuate. Table 2 provides descriptive statistics of the actual measure and system estimation difference either in real value or in percentage from its real value. On average, the difference of both values is 4.982, equal to 19.42% from the actual measure. It is small enough value for a good system's performance. But, just like the case in the comparison between actual measure and its estimation on leaf area, the standard deviation and variance indicating the presence of extreme value. Once again, the median was used to describe the system's performance. The median value is 3.949, equal to 12.09% from the actual measure. Two of the factors cause this extreme value are :

1. Failure in actual chlorophyll intensity measurement during destructive test. Because of its small size, the chlorophyllmetre being used was unable to provide high precision measurement because the leaf can not overlay the sensor perfectly. Hence, the value appear in the device screen is unstable.

2. System's failure in leaf image's pixels identification. In particular condition, the system failed to erase the pixels belong to other object (e.g. branch, root, bottle) such that the *v* component of those object consider belong to leaf.

Those factors also causes the model in equation (6) only explain 12.6% of the actual chlorophyll intensity variation.

TABLE 2
DESCRIPTIVE STATISTICS OF THE ACTUAL AND ESTIMATE DIFFERENCE ON CHLOROPHYL INTENSITY

Statistics	Difference (mm^2)	Difference (%)
Mean	4.982	19.4200
Median	3.949	12.0900
St. Dev	4.148	29.4900
Variance	17.210	869.7300
Minimum	0.030	0.0977
Maksimum	21.605	203.0100
Range	21.575	202.9100

V. CONCLUTION

The system could provide leaf area and chlorophyll intensity estimation with high accuracy. On average, the estimation differences are 19.71% from the actual leaf area measured by millimeter block paper and 12.09% from the actual chlorophyll intensity measured by chlorophyllmetre. Intensity histogram and Fourier spectrum found to be the best feature for sub image selection. Both features could identify 85% of the total correct sub images. In the leaf area estimation, model with pixels amount adjustment due to camera distance variation provide more accurate estimation compared to those without such adjustment. In the chlorophyll intensity estimation, *v* component of the HSV format provides better estimation compared to either *h*, *i*, *s* component of HIS format and G/R ratio or MNDVI index of the RGB format.

FUTURE WORK

1. The system not yet provide automatic leaf high measurement, therefore such information should manually entered by user. With addition of automatic height measurement module into the system, it would perfectly provide automatic and real time analysis.
2. Further study of the correction factor possibility usage is needed to overcome high fluctuation problem in chlorophyll intensity estimation
3. More exploration about camera distance influence on image color is needed in order to increase chlorophyll intensity estimation accuracy
4. Different camera resolution could be tried in image acquisition step to investigate its influence on the morphology measurement performance.

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