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# A Novel Automated Method for Screening Rice Genotypes for Root Growth Rate using ImageJ Software

DONIA ALBERT, KARTHIK NANAIAH AND M. S. SHESHSHAYEE
Department of Crop Physiology, College of Agriculture, UAS, GKVK, Bengaluru - 560 065
e-Mail : doniarani1705@gmail.com

**AUTHORS CONTRIBUTION**

DONIA ALBERT : Conceptualization investigation, original draft preparation and analysis;
KARTHIK NANAIAH : Conceptualization and analysis;
M.S. SHESHSHAYEE : Conceptualization, framed research proposal and draft correction

***Corresponding Author :***

DONIA ALBERT
Department of Crop Physiology,
College of Agriculture,
UAS, GKVK, Bengaluru

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## ABSTRACT

The escalating occurrence and intensity of drought stress due to erratic climate shifts emphasize the significance of understanding plant reactions to such stress, particularly in the context of rice cultivation. Gaining insights into diverse facets of plant response to drought stress is of utmost importance, with a special emphasis on plant roots - the primary organs responsible for extracting water and nutrients from the soil. This study employed the ImageJ software to quantify daily growth in total root length, a task previously unattainable using conventional analysis methods. Leveraging the capabilities of ImageJ, the software automated the estimation of rice root length through the utilization of a well-suited threshold algorithm for image processing. To enhance image analysis through this software, we replaced agar with clerigel as the growth medium. This substitution led to clearer capture of images, consequently facilitating more accurate and precise analysis *via* the software. The integration of computer-based analysis and high-resolution imaging techniques has notably advanced our comprehension of root development and its implications for crop yield and resource management.

*Keywords* : Rice genotypes, ImageJ software, Imaging of roots

Rice is the staple food in Asian countries. It also has high water consumption (Ramachandra *et al.,* 2023 and Prabhu *et al.,* 2023). More than 50 per cent of irrigation water is utilized by rice crops due to their water-intensive nature. Additionally, rice crops are prone to various abiotic stresses such as drought, submergence, heavy metals, salinity and cold. These stresses pose challenges to rice production, especially in the context of global climate change, where drought occurrences are expected to become more frequent (Kim *et al.,* 2020 and Pandey & Shukla, 2015).

To address water scarcity issues, a new system called Aerobic Rice System (ARS) was introduced. The ARS involves growing rice under nonpuddled, nonflooded, and nonsaturated soil conditions, which reduces the need for excessive irrigation water. However, it is noted that this adaptation to reduce water usage in rice cultivation has resulted in a significant reduction in yield compared to traditional methods (Prasad, 2011 and Chauhan *et al.,* 2011).

The major limitations for yield are primarily related to the availability of nutrition and water. Consequently, understanding how plants acquire these essential resources through their root systems has become a fascinating area of research (Lynch, 1995). The root serves as a crucial organ in plants, responsible for regulating shoot growth and overall plant development (Yang *et al.,* 2012). Furthermore, roots play a significant role in enabling plants to adapt to water scarcity conditions (Abobatta, 2019).

During the early stages of seedling establishment, the growth of roots is particularly critical. The total growth of roots in this phase serves as a parameter that helps differentiate between genotypes with varying levels of drought tolerance (Avramova *et al.,* 2016). Therefore, accurately characterizing the architecture of the root system holds great importance in studies related to water-saving rice cultivation, such as the aerobic rice system (Tajima and Kato, 2011). Understanding and phenotyping the root system architecture can provide valuable insights into improving the water-use efficiency of crops, particularly in the context of sustainable agricultural practices.

The advancements in analytical methods have significantly enhanced the assessment of crop root length. Previously, it was highly challenging to measure root growth on a daily basis using conventional analytical techniques. However, with the advent of various algorithms and software, accurate measurement of crop root length has become feasible through computer-based analysis using high-resolution images (Tajima and Kato, 2011).

Tajima and Kato (2011) demonstrated that the software ImageJ exhibited a strong correlation with WinRHIZO in measuring root length. Leveraging this finding, we employed the ImageJ software in our study to determine the daily increase in total root length, a task that was previously unattainable through traditional analytical methods. The ImageJ completely automated the estimation of rice root length with the appropriate threshold algorithm for image processing. To facilitate improved image analysis using the software, we replaced agar with clarigel as the growth medium. This substitution ensured that the images obtained were clearer, enabling more precise and accurate analysis using the ImageJ software.

These advancements in analytical methods and software have revolutionized the field of crop root length assessment, enabling researchers to gather detailed and real-time data on root growth. The utilization of computer-based analysis and high-resolution imaging techniques has greatly enhanced our understanding of root development and its implications for crop productivity and resource management.

## Material and Methods

### Seed Material

In this study, a total of thirty aerobic rice germplasm lines were used, as listed in Table 1. These particular rice genotypes were chosen from the 3K rice panel of the International Rice Research Institute (IRRI) for standardization.

Table 1

Total root length of 30 rice genotypes and primary root length of genotypes measured manually and by software at 6 days after germination

| Genotype | Total root length (cm) | Primary root length (cm) | |
|---|---|---|---|
| | | Manual | Software |
| GEN_RIC_32 | 22.00 ± 1.74 | 3.55 ± 0.08 | 3.35 ± 0.12 |
| GEN_RIC_69 | 16.00 ± 1.19 | 5.05 ± 1.11 | 3.89 ± 0.91 |
| GEN_RIC_75 | 15.00 ± 0.40 | 3.30 ± 0.14 | 3.47 ± 0.29 |
| GEN_RIC_84 | 18.50 ± 0.90 | 3.10 ± 0.14 | 2.84 ± 0.11 |
| GEN_RIC_85 | 23.17 ± 1.57 | 4.55 ± 0.50 | 3.76 ± 0.57 |
| GEN_RIC_144 | 34.09 ± 3.19 | 3.65 ± 0.05 | 2.41 ± 0.21 |
| GEN_RIC_156 | 16.12 ± 0.78 | 2.45 ± 0.26 | 2.13 ± 0.04 |
| GEN_RIC_189 | 16.70 ± 0.59 | 4.37 ± 0.80 | 4.63 ± 0.23 |
| GEN_RIC_204 | 24.50 ± 3.02 | 4.50 ± 0.17 | 4.64 ± 0.05 |

**Table 1 Contd...**

| Genotype | Total root length (cm) | Primary root length (cm) | |
|---|---|---|---|
| | | Manual | Software |
| GEN_RIC_216 | 21.00 ± 0.20 | 2.90 ± 0.47 | 2.75 ± 0.31 |
| GEN_RIC_222 | 7.51 ± 0.75 | 3.00 ± 0.08 | 2.71 ± 0.08 |
| GEN_RIC_238 | 21.30 ± 1.35 | 5.15 ± 0.29 | 3.39 ± 0.15 |
| GEN_RIC_249 | 13.45 ± 1.27 | 2.25 ± 0.59 | 2.24 ± 0.24 |
| GEN_RIC_254 | 8.00 ± 0.76 | 2.13 ± 0.26 | 2.17 ± 0.18 |
| GEN_RIC_319 | 9.68 ± 0.87 | 4.25 ± 0.56 | 4.34 ± 0.15 |
| GEN_RIC_325 | 10.90 ± 0.88 | 3.50 ± 0.09 | 3.94 ± 0.25 |
| GEN_RIC_346 | 22.80 ± 1.61 | 3.60 ± 0.16 | 3.63 ± 0.25 |
| GEN_RIC_358 | 13.69 ± 0.54 | 3.80 ± 0.09 | 3.47 ± 0.11 |
| GEN_RIC_394 | 5.15 ± 0.73 | 3.45 ± 0.14 | 3.37 ± 0.09 |
| GEN_RIC_470 | 17.47 ± 1.41 | 3.47 ± 0.40 | 3.65 ± 0.18 |
| GEN_RIC_498 | 14.30 ± 0.29 | 2.75 ± 0.21 | 2.69 ± 0.09 |
| GEN_RIC_518 | 24.50 ± 0.09 | 4.30 ± 0.29 | 3.72 ± 0.05 |
| GEN_RIC_521 | 13.90 ± 0.92 | 3.00 ± 0.59 | 3.11 ± 0.12 |
| GEN_RIC_531 | 28.38 ± 1.12 | 4.95 ± 0.45 | 4.19 ± 0.14 |
| GEN_RIC_584 | 19.39 ± 1.42 | 3.75 ± 0.12 | 3.67 ± 0.15 |
| GEN_RIC_608 | 8.82 ± 0.25 | 3.40 ± 0.21 | 3.32 ± 0.07 |
| GEN_RIC_612 | 23.50 ± 0.53 | 5.30 ± 0.16 | 3.49 ± 0.16 |
| GEN_RIC_621 | 23.80 ± 0.28 | 3.00 ± 0.00 | 3.00 ± 0.05 |
| GEN_RIC_733 | 30.80 ± 0.69 | 3.53 ± 0.09 | 3.20 ± 0.08 |
| GEN_RIC_755 | 6.49 ± 0.41 | 1.63 ± 0.45 | 1.57 ± 0.08 |

**Sterilization of Seed**

To ensure successful germination and subsequent experimentation, the seeds of the selected rice genotypes underwent several preparatory steps including sterilization of seeds by subjecting to 0.1 per cent Bavistin solution. This sterilization step aimed to prevent any potential fungal infections that could hinder the growth and development of the seeds (Vasanthakumari *et al.,* 2019). Following the sterilization treatment, the Bavistin-treated seeds were thoroughly rinsed three times using sterile water. This rinsing process aimed to eliminate any excess Bavistin residue that might have remained on the seeds after the sterilization treatment. Removing the excess Bavistin was important to ensure that it would not have any adverse effects on the germination process or subsequent growth of the rice plants (Gaind *et al.,* 2007).

After the sterilization and rinsing steps, the seeds were germinated. To facilitate this process, the sterilized seeds were placed on blotting sheets within petri plates. These blotting sheets served to maintain a moist environment around the seeds, providing the necessary conditions for germination to occur. The petri plates offered a controlled and contained setup for observing and monitoring the germination progress of the rice seeds.

**Growth Condition**

The uniformly germinated seeds were chosen to ensure consistent and reliable results. To provide an appropriate growth environment for the germinated seeds, the seeds were placed in test tubes filled with solid clarigel medium, supplemented with half strength MS (Murashige and Skoog) to provide support and nutrients to the growing seedlings (Kaur and Kumar, 2020). Three replications for each genotype were maintained. To maintain optimal growth conditions and promote consistent development, the test tubes containing the seedlings were placed in a growth chamber. By maintaining uniform levels of light and temperature, external factors that could influence the growth and development of the seedlings were minimized. The seedlings were allowed to grow for a specific duration of six days. This time frame was chosen to observe and evaluate the growth and morphological characteristics of the root within a defined period.

**Imaging of Roots**

The root imaging involved capturing digital images of root growth starting from the first day after incubation in a test tube. Day 0 was designated as the day when the seeds were initially placed. Subsequently, the root growth was documented daily from a fixed distance until day six. To ensure comprehensive coverage and accurate measurements of all the growing roots, photographs were taken from all sides of the tubes, considering that roots grow in multiple directions within the tube.

**Estimation of Total Root Length Using ImageJ**

After capturing the root images, the length of both the minor and major roots were analyzed using the ImageJ software. To begin the analysis, the images were opened from the toolbar. Before measuring the root length, a standard scale was established using measuring scale placed near the test tube containing the seedlings. In this experiment, the scale was set to 1 cm (Fig. 1a) and the corresponding number of pixels covered by the 1 cm scale was manually assigned as 1 unit (Fig. 1b). Once the standard scale was set, the actual measurements were conducted using the segmented line tool in the tool bar. A line was traced on the roots using this tool (Fig. 2). After tracing each root to the desired length, the software automatically measured the length of the traced line. The secondary and minor roots was also considered to derive the total root length. Total root length was measured is sum of all the roots (Primary, secondary and minor roots) measured by the software. Relative growth of root was calculated by plotting the daily increase in total root length.

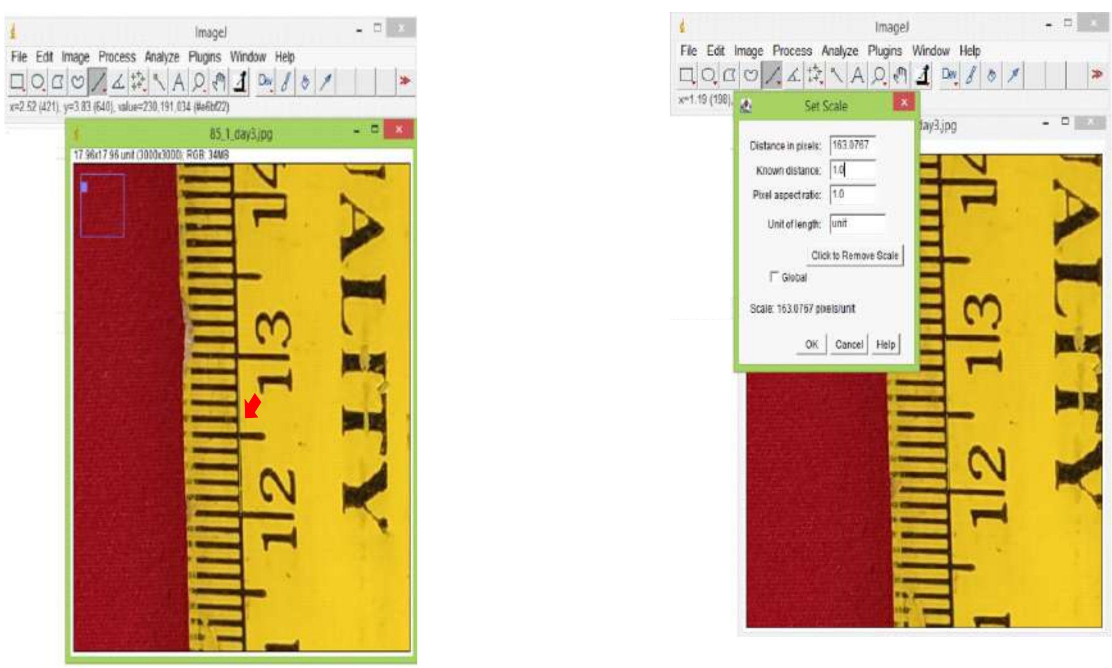


Fig. 1: Image depicts the setting ofstandard scale using the measuring scale. a) Line drawn on the scale using straight line tool. Red arrow indicates the line drawn on the scale b) the scale was set as 1cm in the known distance so that number of pixels covered by the known distance will be measured by the software.

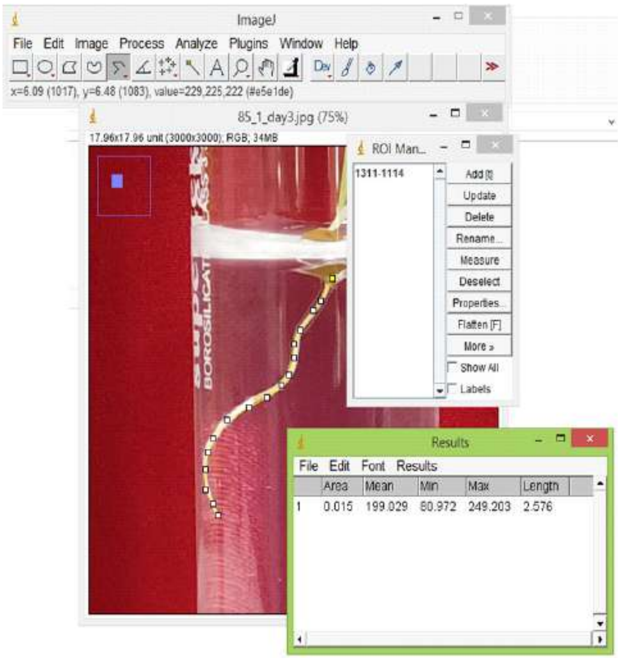


Fig. 2: Image showing line drawn on the root using segmented line tool and measured root length by the software

### Estimation of Root Length Manually

To confirm the accuracy of the root length observed using the ImageJ software, manual measurements were conducted. After a period of six days, the roots were uprooted from the Clerigel without causing any harm or cuts. The length of the primary root (the longest root) was measured manually using a measuring scale.

## Results and Discussion

### Total Root Length Variation Among Different Genotypes

Total root length of all the genotypes differed significantly. Among the thirty genotypes, GEN_RIC_394 has the lowest total root length (5.15 cm) and GEN_RIC_144 has the highest total root length (34.09 cm) (Table 1). The average total root length was 17.6 cm.

### Difference in Total Root Length Between the Groups

Root growth was calculated on daily basis for six days for all the 30 rice genotypes. All the genotypes showed diversity in root traits. Genotypes were categorized into minimum, medium and maximum root types based on their total root length. Genotypes with a total root length ranging from 5 to 10 cm were classified as minimum root types, those with a length between 13 and 20 cm were grouped as medium root types and genotypes with a length exceeding 20 cm were considered maximum root types. Box plot analysis shows that there is a significant difference between total root length of the minimum, medium and maximum root types (Fig. 3).

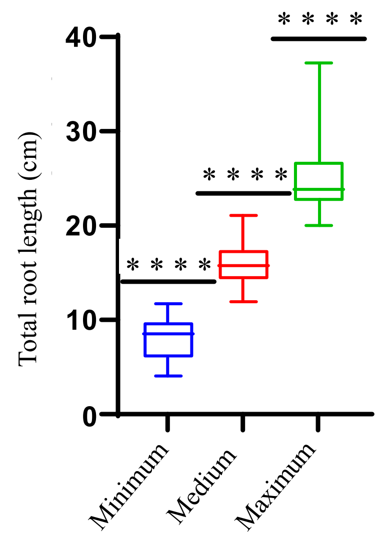


Fig. 3 : Box plot showing difference between total root length of minimum, medium and maximum root genotypes. One-way ANOVA was carried out to determine significance between minimum, medium and maximum root genotypes (****P<0.0001).

### Correlation Between Manual Measurement and Software

Primary root (first formed root) length measured using the software Image J was almost similar to that of manual method (Table 1). For the genotypes GEN_RIC_254, GEN_RIC_346 and GEN_RIC_621, the primary root length measured manually and by the software ImageJ was same(Table 1). A strong positive correlation between the primary root length measured by the ImageJ software and manual measurement was observed in all the genotypes (Fig. 4).

### Relative Root Growth

After day four, the majority of genotypes exhibited a more pronounced increase in root length. However,

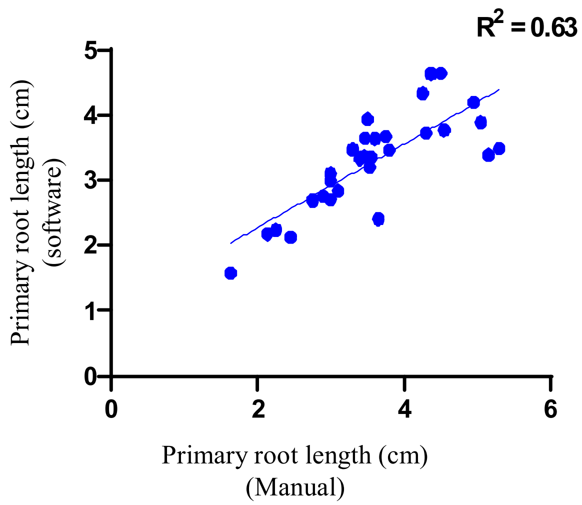


Fig. 4: Graph showing correlation between the primary root length measured by Image J software and manually

in genotypes with the minimum total root length, the steeper increase was observed after day three, with the genotype GEN_RIC_394 displaying a comparatively lower steepness compared to other genotypes (Fig. 5a). Until day four, there was a gradual increase in root length, but after that, both medium and maximum root types exhibited a more substantial increase. Prior to day four, seedlings only possessed a primary root, but after day four, lateral roots started to emerge from the primary root (Fig. 6). The emergence of lateral roots resulted in an observed increase in the total root length. (Figs. 5b and 5c)

The ability of the plant to absorb water and minerals from the soil and providing resistance against various abiotic stresses in many crops is federated with its root system architecture (RSA) (Koevoets *et al.,* 2016). Plants with higher root system forms an ideal candidate to get more yield, abiotic stress tolerance and faster growth (Wang *et al.,* 2020). Hence identification of genotypes with higher root growth could help for breeding of new varieties with resistance to several environmental conditions.

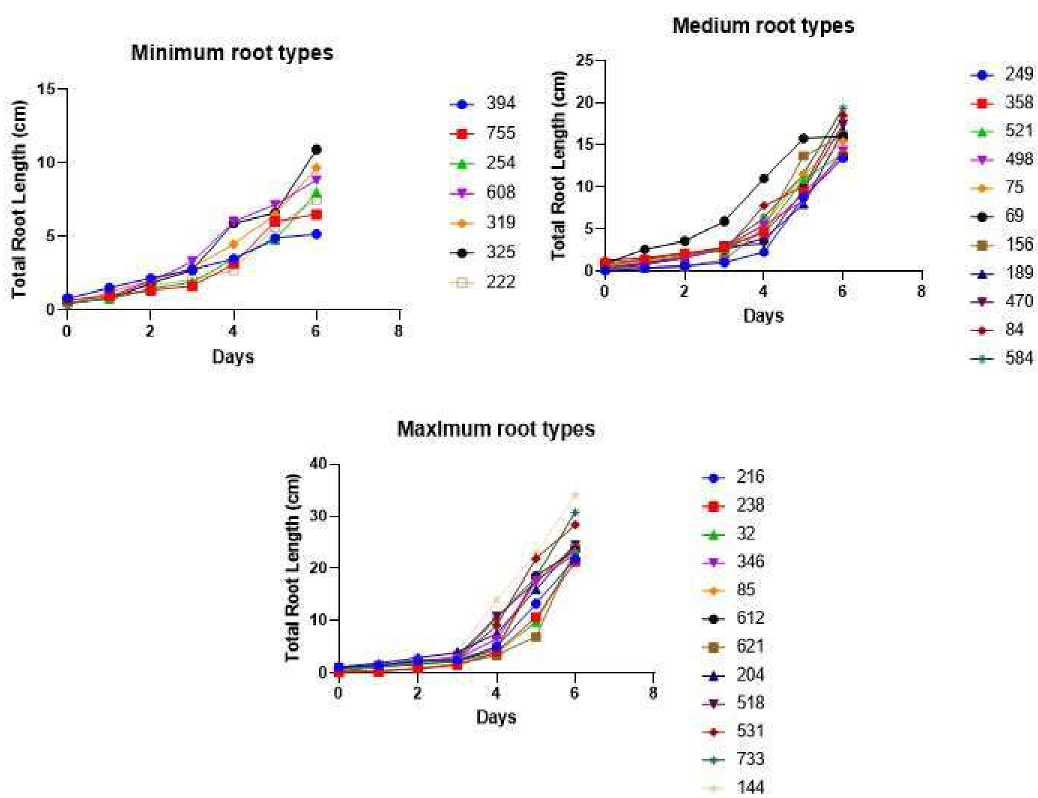


Fig. 5 : Root growth on daily basis of a) minimum root type genotypes; b) medium root type genotypes c) maximum root type genotypes


Fig. 6 : Digital image showing daily growth of root form day 0 to day 6

The destiny of seedlings will determine the performance of most plant species (De La Cruz *et al.,* 2008). Hence, evaluating the phenotype at seedling stage is easy, low cost and time saving method (Li *et al.,* 2015), but it is difficult to measure daily increase in root length under soil condition. Several methods have been developed for screening of rice genotypes for root traits including aeroponics, hydroponics and agar plate system. For this study, the seedlings were grown in transparent media and the daily fluctuations in root growth using ImageJ software was examined. ImageJ is an open-source software widely employed in various image analysis technologies (Schneider *et al.,* 2012). By integrating digital camera technology, image analysis software (ImageJ) and transparent media, the total root length on a daily basis was assessed. Maintaining a consistent growth period for the seedlings has allowed to effectively compare and analyze the impact of experimental conditions on the plants at a comparable developmental stage.

**The Primary Determinant for Variability in Total Root Length Lies in Secondary Root**

The relative growth rate of root was slower up to four days after germination and after fourth day the root length was higher. In the total root system lateral roots forms the greater proportion in total root length and root number (Banoc *et al.,* 2000). Our results shows that the lateral roots started to develop from the primary root after day four which may be the reason for increase in the total root length after day four. In this experiment the genotypes were categorized into three groups based on their total root length as minimum, medium and maximum root types. Maximum root types are better for high WUE and nutrient uptake. Here, GEN_RIC_733, GEN_RIC_144 and GEN_RIC_531 showed higher root length. These genotypes had more lateral roots. Lateral root formation is formed as a result of auxin biosynthesis, polar auxin transport and auxin degradation / conjugation change auxin accumulation and auxin-dependent signaling (Zhang *et al.,* 2018). Therefore, these genotypes might have more endogenous auxin signaling events happening which lead to the formation of more lateral roots and therefore the total root length. As Wang *et al.,* 2020 assessed genotypes with higher root system forms a best system for producing higher yield and tolerance to abiotic stress, these genotypes could be used in breeding programs for developing better performing rice varieties (Figs. 3 and 4).

**Correlation Between Manual Measurement and Software Emphasizes the Necessity of Reliable Digital Tools**

Tajima and Kato, 2011 showed that Image J is a best software for measuring root parameters using the root images. In current experiment, we obtained a strong correlation between the manual method and ImageJ software based measurement of root length which confirms that ImageJ is one of the best software to measure the root length (Fig. 5). Root growth is influenced by various factors and one of the significant determinants is the relative root growth, which refers to the comparative rate of growth between different roots within a plant or between genotypes. The concept of relative root growth encompasses the idea that the growth of one root system can impact the growth of other roots or even the entire plant. This interplay between roots can be influenced by a multitude of factors, including nutrient availability, water availability, soil structure, hormone distribution and interactions with neighboring roots. Consequently, the relative root growth becomes a crucial aspect to consider when studying plant development, as it can shape the overall architecture, efficiency of nutrient uptake and the ability to withstand environmental stresses. Understanding the complex dynamics of relative root growth holds significant implications for optimizing crop production, enhancing plant resilience and improving sustainable agricultural practices.

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