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Determination of age-dependent endogenous indole-3 acetic acid (IAA) level in different organs of tomato plants

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ABSTRACT

Indole-3-acetic acid (IAA), a phytohormone, is a crucial modulator of plant growth and development. As increased levels of endogenous IAA is responsible for inhibitory effects on plants, knowing its concentration would be helpful for controlling exogenous IAA input. An optimum endogenous IAA level is required for proper growth and development of plant with good health status. Hence, in this study, we have detected and measured IAA present in tomato root, shoot, leaves and fruits at two different ages (25 days and 50 days) by Reversed-Phase High Performance Liquid Chromatography (RP-HPLC), a suitable chromatographic method for separating molecules based on hydrophobicity. We observed that the endogenous level of IAA was significantly higher in roots, shoots, leaves and fruits at 50-days old plant compared to 25-days old mature plant (p-value <0.01). These results suggested that the IAA level may increase with the increase of the age of tomato plants. The tomato plants showed good vigor with IAA range 0.3-3.0 µg/g.F.W (25 days) and 3.4-7.5 µg/g.F.W (50 days) of age, respectively, implying that IAA might be used as a possible plant health indicator. Further study is needed to manipulate the IAA concentration for adequate plant growth and development.

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1. Introduction

Tomato (*Lycopersicon esculentum* Mill.) is a widely grown vegetable in many countries around the globe for its nutritional value since it acts as a rich source of various antioxidants (Frusciante et al., 2007). Its proper and healthy growth is essential for maximum yield. Various plant growth regulators have been involved in growth and development. Among them, Indole Acetic Acid (IAA), the most abundant plant hormone of the auxin group, plays an important role in improving the plant growth and yield of tomato plants. IAA is produced in young leaves and cotyledons as a conjugated complex with amino acids, sugars or remain at free state (Olatunji et al., 2017). It promotes adventitious root formation, embryogenesis, seedling growth, vascular patterning and flower development (Guan et al., 2019). As an intercellular signalling molecule, IAA couples environmental stimuli to plant growth responses in phototropism and gravitropism (Nakamura et al., 2019). It can keep the leaf stomata open by inhibiting the activity of another phytohormone (Sun and Li, 2014). Endogenous IAA is regulated by biosynthesis, conjugation and catabolism (Olatunji et al., 2017). The activity of IAA can be modulated by altering its endogenous concentration or the sensitivities of target tissues or organs towards it (Kim et al., 2006). Various plant pathogens secret IAA, enhancing endogenous IAA levels in plants and causing diseases, including necrotic lesions and tumour. In plants, elevated IAA concentrations inhibit seed germination, plant growth and impede salicylic acid pathway leading to biotrophic pathogen infection (Bunsangiam et al., 2021; Duca et al., 2014). In order to maintain optimum endogenous IAA level in plants and to avoid excess exogenous IAA application, IAA quantification is more than the necessity. Different high-throughput techniques have been used for IAA detection and measurement. Among them, reversed-phase HPLC is a better option for IAA analysis concerning resolution, chromatographic reproducibility, faster equilibration of mobile phase, sensitivity and cost (Du et al., 2012; Leonard and Traber, 2019). It is appropriate for isolating biomolecules which have non-polar moieties as IAA (Nakurte et al., 2012). Plant hormone level can be changed with age as the shoots and leaves grow and mature till senescence. Previously, study on age-dependent endogenous IAA level in different organs of tomato plant was not undertaken. In addition, there is a knowledge gap regarding optimum IAA level that ensures plant good health and efficient development at various ages. In this study, we have determined endogenous IAA level present in tomato root, shoot, leaf and fruit at 25 and 50 days after planting (DAP) and checked level of IAA in healthy tomato plant. This study is necessary to find out the proper endogenous IAA level in a healthy tomato plant ensuring optimum growth and development.

2. Materials and methods

2.1. Preparation of standard IAA solution

The IAA standard used in the experiment was commercially purchased as free IAA (Sigma Aldrich, USA). The standard stock solution of 1000 mg/L was prepared by dissolving 0.01 g standard IAA in 100 % methanol (HPLC grade). It was serially diluted to 1 mg/L. From 1 mg/L each of the working solutions- 50, 100, 200, 300, 400 and 500 µg/L were prepared. Against these concentrations of IAA standards, the HPLC peak areas were plotted. The standard calibration curve (Fig. 1) showed linear regression and regression coefficient, R^2=0.989, indicating that the method could be used for IAA detection and quantification.

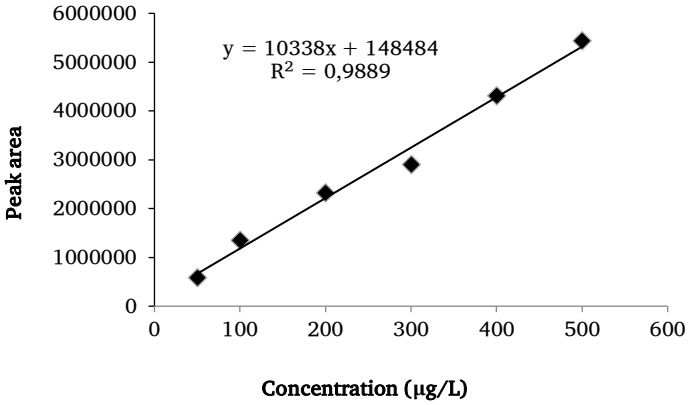

Fig. 1. Standard calibration curve for IAA determination at 360 nm emission

2.2. Collection of plant materials

Tomato seeds were sown in a net house under environmental conditions (20-25°C temperature and 6/18 h day/night photoperiod). The seeds were germinated within 2-3 days. Germinated seedlings were transferred from the seedbed to pots. After 25 and 50 DAP, which were chosen as examples of two different developmental stages of tomato life cycle, entire plants were harvested and cleaned. Root, shoot, leaves and fruits were chopped into small pieces from each plant and weighed.

2.3. IAA extraction from plant material

The plant samples were ground using cold methanol (4°C) in a mortar-pestle. The samples were centrifuged (6,000 rpm, 20 min) at 4°C. Methanol was evaporated and pure water was added in order to enhance the polarity of the sample. The pH of the plant samples was adjusted to >10 using KOH (1 M) followed by the separation against 100% ethyl acetate (Sigma Aldrich, USA) and the aqueous phase was collected after centrifugation (10,000 rpm, 10 min). The pH of the solution was attained to <3 with concentrated acetic acid (>99%, Sigma Aldrich, USA). Then the acidic sample was partitioned against ethyl acetate and cleared by centrifugation at 4000 rpm for 10 min again. The organic phase was collected and completely dried in an evaporator. Finally, it was dissolved in a marginal volume (2 ml) of methanol (Sigma Aldrich, USA).

2.4. Purification of IAA extract

For purification, 0.15 g Primary Secondary Amine (PSA) (Supeclo, USA) and 0.25 g Octadecylsilyl (C18) (Agilent, USA) were added to the dissolved the plant extract. The mixture was shaken for 1 min using vortex, homogenized, centrifuged for 5 min at 2000 rpm and finally filtered through a membrane filter (0.22 µm) prior to RP-HPLC.

2.5. Determination of IAA concentration by RP-HPLC

IAA concentration of different organs of tomato plants were measured in two different stages of tomato life cycle, i.e., before flowering (25 DAP) and at fruit-bearing stages (50 DAP). Chromatographic determination of IAA standards and samples were performed using RP-HPLC machine (Prominence, Shimadzu) with C18 column (shim-pack GWS 4.6x150 mm). The mobile phase was composed of methanol and deionized water containing 1% acetic acid at a volumetric ratio of 60:40 in isocratic mode (Nakurte et al., 2012). Several parameters were fixed prior to program operation. The temperature of the column oven (CTO-10AS VP) was set at 30 °C. The pump flow rate was 1 ml/min and program run time was set to 7 min. For the sample and standard, 20 µL was the injection volume. The presence of IAA was detected by the fluorescence detector (RF-20A Prominence fluorescence) with an excitation at 282 nm and emission at 360 nm. Results were analyzed by Lab Solution (Shimadzu) software.

2.6. Validation of RP-HPLC method

RP-HPLC method was validated in terms of accuracy determination. For accuracy, recovery of each extracted sample type, i.e., root, shoot, leaf, fruit of 25 DAP and 50 DAP was measured at 250 µg/L. The percent (%) recovery was calculated using the following formula:

$$Recovery\ (\%) = \frac{peak\ area\ of\ spiked\ samples - peak\ area\ of\ unspiked\ samples}{peak\ area\ of\ standard} \times 100 \quad (1)$$

2.7. Statistical analysis

Statistical analysis was performed using SPSS version 24 (USA) and MS office. F-test and t-test was done at 99 % confidence interval between 25 days old and 50 days old tomato plants in terms of concentration and content of IAA extracted from root, shoot, leaf and fruits.

3. Results and discussion

3.1. Results

By comparing the retention time and peak area of the standard free IAA, the presence of free IAA in the samples were detected and quantified (Fig. 2, Table 1). The average retention time for the standard was 3.27±0.01 min. The samples also showed a peak at a similar time indicating the detection of IAA (Table 1).

Table 1. The mean retention time of IAA detected from standards and samples

Sample type	RT ± SD (min)
Standard	3.27±0.01
25-days root	3.32±0.05
25-days shoot	3.26±0.08
25-days leaf	3.29±0.04
50-days root	3.34±0.01
50-days shoot	3.33±0.07
50-days leaf	3.33±0.03
50-days fruit A	3.31±1.4
50-days fruit B	3.30±0.02

Note: RT= mean retention time, SD = standard deviation, s=second. For each sample type N=18, A and B were two different weights of tomato fruits

The equation is derived from the standard IAA calibration graph, $y = 10338x + 148484$ (Fig. 1) was used to quantify the IAA concentration in the extracted and purified plant samples. Concentration was calculated at µg/L level per tomato plant (Table 2) using the peak area obtained for the corresponding retention time. In 25-days old plants, the concentration of IAA per plant was the highest in the leaf (242.1±0.8 µg/L) and lowest in the shoot (28.0±1.0 µg/L). In the case of root, the concentration level was in-between those of the shoot and leaf (42.0±1.0 µg/L). In 50-days old plants, the order of IAA concentration in the tomato samples was fruits<shoot<leaf<root.

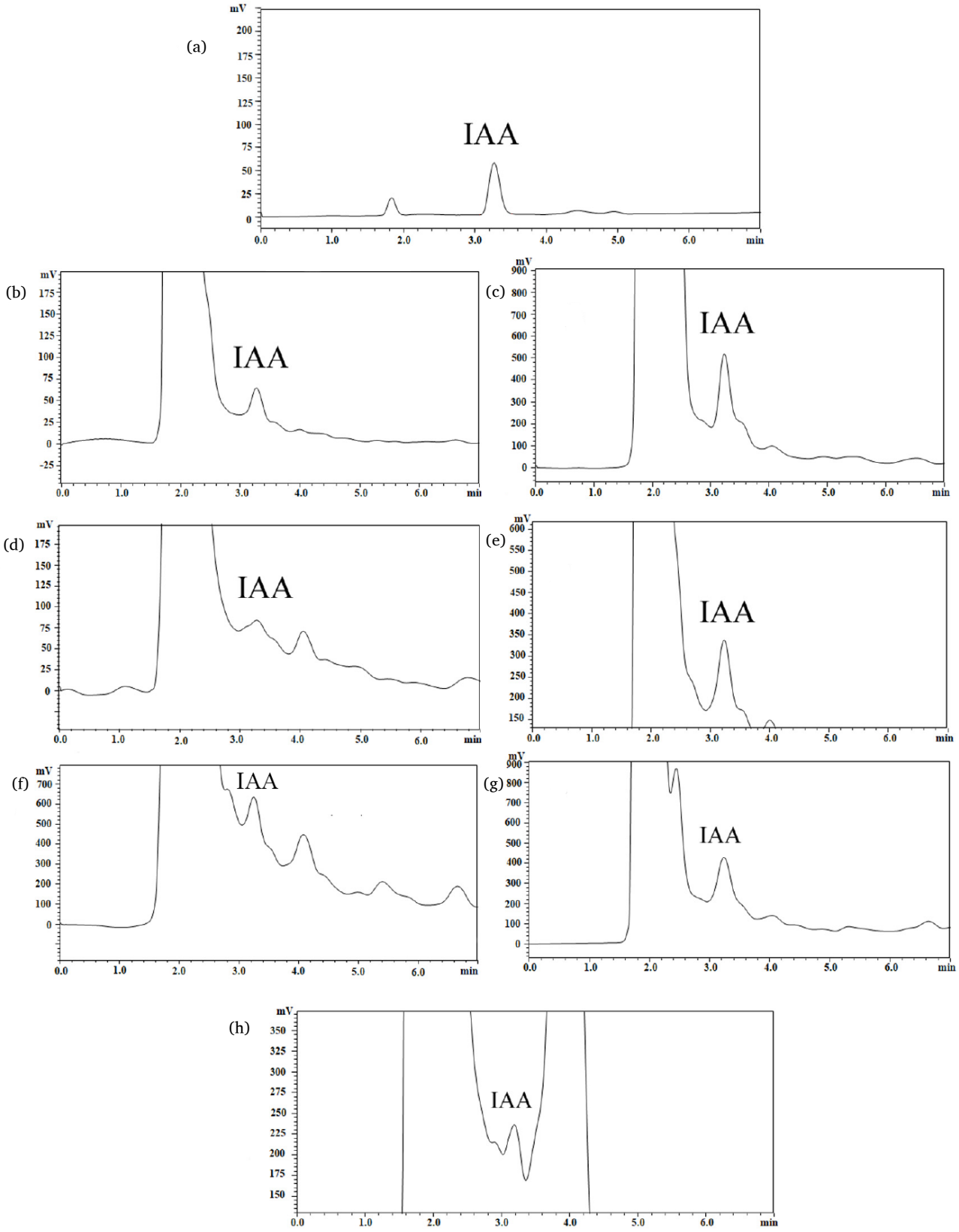

Fig. 2. RP-HPLC Chromatogram of (a) IAA standard (50 µg/L) and (b-h) the samples. IAA detected in 25-day old tomato (b) root, (d) shoot, (f) leaf and 50-day old tomato (c) root, (e) shoot, (g) leaf, (h) fruit. Along x axis=retention time and y axis= fluorescence signal at 360 nm (mV)

A significant difference ($p<0.01$) was found between 25-day old mature, but no flowering plants and 50-day old fruiting tomato plants in terms of IAA concentration and content in the root, shoot, leaf and fruits. IAA content was higher in 50-day old plant compared to the 25-day one (Table 2, Fig. 3). Data represented as mean±standard error, n=6. a,b,c,d=significant difference between 25-day and 50-day old root, shoot, leaf, fruit; p-value <0.01. A and B were two different weights of tomato fruits. The morphological features of the tomato plants took in this study contained higher IAA values and showed good health (Fig. 4). So the endogenous IAA

status regarding root-leaf, 0.3-3.0 µg/g.F.W for 25 days and 3.4-7.5 µg/g.F.W for 50 days aged plants might be used as an indicator of good vigor and development of tomato plants of the corresponding age. Hence, it is notable that IAA together with the nutrients can boost up the healthy properties of plants.

Table 2 Level of IAA in root, shoot, leaf and fruits of each tomato plant at two stages of life cycle

Plant age	Sample type	Sample weight/ plant (g)	IAA concentration /plant (µg/L)	IAA content/plant	
				µg/g.F.W.	nmol/gFW
25-days	root	7.0±0.1	42.0±1.0	[a]0.3±0.01	1.65±0.04
	shoot	21.0±0.4	28.0±1.0	[b]0.6±0.02	3.3±0.1
	leaf	12.1±0.6	242.1±0.8	[c]3.0±0.01	17±0.1
	fruit	0	0	[d]0	0
50-days	root	16.5±0.3	452±1.9	[a]7.5±0.1	43±0.6
	shoot	20.1±0.3	320±1.4	[b]6.4±0.08	37±0.4
	leaf	9.5±0.7	355.1±1.8	[c]3.4±0.2	19.2±1
	fruit A	3.5±0.5	22.4±1.4	[d]0.1±0.01	0.4±0.1
	fruit B	8.5±0.7	51.5±1.2	[d]0.44±0.03	2.5±0.2

Note: µg/g.F.W= micro gram/gram fresh weight;nmol/gFW = nano mole/gram formula weight

The accuracy of RP-HPLC was determined by recovery calculation using formula (1). Twenty-five-day old extracted tomato plant samples showed 90-116% recovery in leaf-to-root. On the other hand, 50-day old plant samples had the recovery range 108-119.4 % in root-to-fruit indicating the accuracy of the RP-HPLC method.

3.2. Discussion

IAA stimulates root and stem development, lateral root initiation, root elongation by increasing cell division and extension (Lam et al., 2020). The tomato plants subjected to this research were at the mature stage (25 days) and the fruiting stage (50 days). Our results showed that IAA content was higher in leaf compared to shoot in both 25-day and 50-day old plants. It was previously found that IAA is produced in elongating shoot apical meristem actively and then transported to the shoot cambium, where it induces tracheid production (Kijidani et al., 2014). The level of IAA in shoot apical cells decreases when leaf primordia expand (Avsian-Kretchmer et al., 2002) and also there is a trend of IAA increase in expanding, newly mature, matured and aged leaves until senescence (Terry et al., 1986).

The possible explanation is that, at the mature stage of tomato plants, the shoots might stop elongation but the leaf branches bear many expanding, mature and aged leaves due to the synthesis of IAA in the shoot apical meristem and polar auxin transport to the leaves from the shoots. Hence there is a possibility of finding less IAA content in the shoot, but more in the leaves. IAA values were reported significantly higher in fruiting plants compared to those of non-flowering ones (Yan et al., 2019). In this study, a similar fashion was observed. IAA is the predominant auxin found in young fruits (Pattison et al., 2014) and contributes to fruit development and maturation.

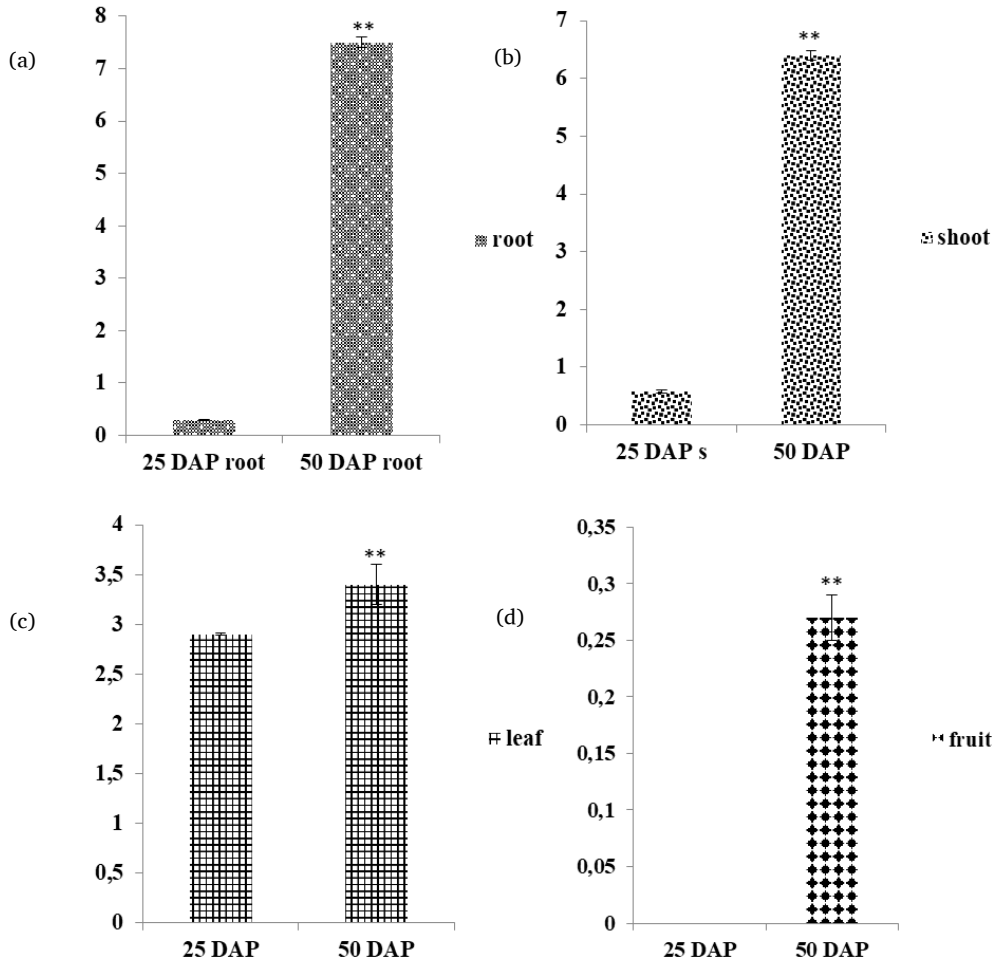

Fig. 3. IAA content in different organs (a) root, (b) shoot, (c) leaf and (d) fruit of tomato plants after 25 days and 50 days of planting. Along y axis= measurement of IAA per plant in microgram/ gram fresh weight (µg/g. F.W). DAP= days after planting. ** =Significant difference of IAA values per plant between 25 DAP and 50 DAP in different plant compartments (root, shoot, leaves and fruit, p-value <0.01)

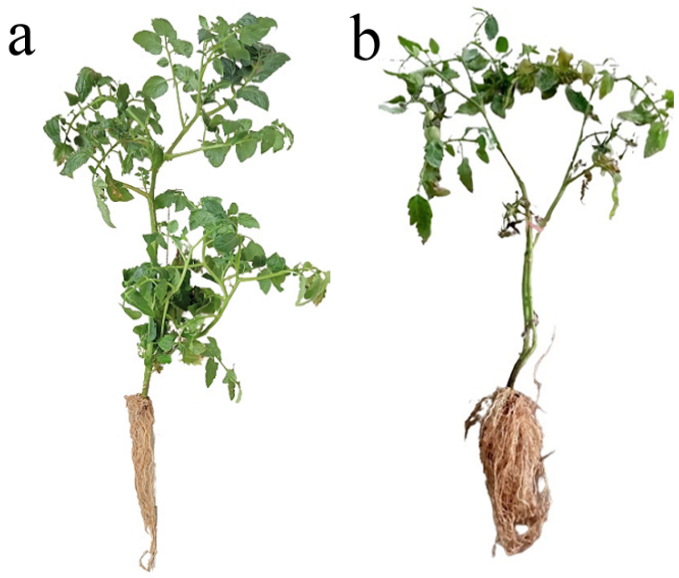

Fig. 4. Photograph of a representative tomato plant of (a) 25 days old mature (IAA: root-leaf=0.3-2.9 µg/g.F.W) and (b) 50 days old fruiting (IAA: root-leaf=7.7-3.4 µg/g.F.W) stage.

IAA concentration gradient changes over time towards a particular developmental stage. Variation of endogenous IAA levels at different ages of tomato plants occurs due to many reasons. Firstly, IAA oxidase and peroxidase cleave IAA in older plants and decrease IAA levels (Ghosh et al., 2015). Secondly, at a greater physiological age, IAA accumulation can be increased in different organs revealing reduced auxin efflux in those tissues. Transporter sensitivities to IAA may vary due to the inactivation of the transporter genes by mutation. Thirdly, Defects in IAA responsive genes can result in lower levels of IAA leading to reduced root density (Wen et al., 2014). Finally, other phytohormone-dependent gene expressions in response to seasonal changes can regulate IAA synthesis and function at various growth stages (Yan et al., 2019).

When the standard IAA (20 µL) is injected via the hamilton syringe into the rheodyne injector, after a certain time it gives a peak in the chromatogram and the time is known as retention time. The retention time of standard IAA and samples are compared in each chromatogram for identification of IAA in samples. A known amount of standard IAA was also added in tomato and analyzed following the same extraction procedure and was injected into HPLC system for recovery. The percentage of recovery was calculated by the amount of spiked sample minus the amount of unspiked sample divided by adding standard's concentration then multiplying by 100. The recovery percentages of spiked samples in our study were 70-120% which was within the acceptable limit of CODEX Alimentarius (2017) indicating the trueness of method validation.

IAA level found in tomato plants measured in this research is higher than IAA level found previously in maize (Kim et al., 2006). Furthermore, use of IAA range that we proposed for tomato plant in this study as a marker of good plant vigor is not previously reported. High IAA reduces plant growth due to the occurrence of ion imbalance at higher concentrations (Kijidani et al., 2014). If endogenous IAA concentration is known, external IAA input can be adjusted avoiding overuse of IAA. Also, the endogenous auxin level can be altered to promote organogenesis.

4. Conclusion

This study reveals that IAA concentration increase with the age of tomato plant. Degree of IAA content varies at different plant compartments along with the stages of development. The identified IAA range, root to leaf:0.3-2.9 µg/g.F.W in 25-days old plant and root to leaf:7.7-3.4 µg/g.F.W in 50 days old tomato plant manifested good plant vigor and therefore can be set as indicator following further experimentation and reproducibility assessment. Impact of IAA on the growth and health status of tomato and other crops should be further investigated.

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Conflict of Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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