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An efficient approach of *in vitro* plant regeneration and propagation of mungbean [*Vigna radiata* L. (Wilczek)]

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ABSTRACT

Mungbean [*Vigna radiata* L. (Wilczek)] is a valuable legume crop in Bangladesh. However, it faces severe difficulties forming viable roots during the growth stage. The successful root production of mungbean through an efficient tissue culture system has not been well established. Therefore, this study aims to investigate the problem of unsuccessful root formation in mungbean plants and to develop a very efficient method of *in vitro* regeneration using micropropagation. The BARI Mung-3 mungbean variety was used as an explant. Several shoots were produced from cotyledonary node (CN) explants obtained from 3-day-old seedlings that were germinated *in vitro*. The plant samples were cultivated on MSB5 medium enriched with 5.0 μM BAP. Shoot generation efficiency per plant was 5.36 ± 0.56 (80.08%). However, the viable root generation in a regulated setting was unsuccessful despite employing multiple combinations of rooting media, including full and half-strength MSB5 medium with different concentrations and combinations of auxins. To resolve this issue, a micrografting approach was applied with scion 3.0 cm in length and 14-day-old mungbean rootstocks. This system resulted in efficient shoots where the viable root generation efficiency rate was 55%. Interestingly, the micro-grafted plantlets successfully produced viable seeds. The successful micropropagation with viable root generation in mungbean plants successfully overcomes the difficulties in rooting and offers an efficient method for successful mungbean production. These new findings open new options for efficient plant generation with mass propagation for ready-smart mungbean production.

INTRODUCTION

Mungbean [*Vigna radiata* L. (Wilczek)], is commonly called green gram and is considered a highly significant pulse crop globally. This organism belongs to the Fabaceae family and the Papilionaceae subfamily. Legumes are regarded as the second most considerable food crop for humans. Legume seeds are a vital component of the human diet due to their high content of proteins, bioactive compounds, minerals, and vitamins. Compared to grains, legumes are often referred to as "the poor man's meat" [1]. This legume crop is highly significant and widely cultivated, occupying over 6 million hectares globally (around 8.5% of the total pulse area). It is a staple in Asian families [2]. The mung bean is rich in a well-balanced combination of nutrients, such as protein, dietary fiber, minerals, vitamins, and substantial quantities of bioactive substances [3]. Mungbean grains are also widely popular for their high digestibility and good flavor. Mungbean sprouts are regarded as a vegetable high in vitamin C and contain 12 times as much iron as regular Mungbean seeds [4]. Nevertheless, the productivity of Mungbean varieties grown in various regions worldwide varies, and their potential yields are known to be influenced

by factors such as environmental conditions, pests and diseases, inadequate farming techniques, and limited access to high-quality seeds of new varieties for farmers [5]. The main factor contributing to reduced crop yield is its vulnerability to several diseases, including yellow mosaic virus, *Cercospora* leaf spot, powdery mildew, and root rot [6, 7]. The Mungbean yellow mosaic virus (MYMV) is regarded as the most destructive disease of Mungbean in South Asian countries, especially Bangladesh [8, 9]. Conventional breeding produced limited success in obtaining virus disease resistance in Mungbean. Thus, it is hypothesized that the enhancement of this significant crop can be accomplished by employing advanced biotechnology techniques, such as plant genetic engineering. It is widely acknowledged that the successful implementation of plant genetic transformation requires an effective *in vitro* plant regeneration system. Mungbean is recalcitrant and the number of regenerating shoots remained very low and limited to a few only, That's why suitable tissue culture methods are needed for the improvement of Mungbean through micropropagation [10].

In the past, several attempts have been made to establish a suitable, effective reproducible *in vitro* regeneration protocol for Mungbean using different explants [11-16]. Developing such regeneration has been reported to be more difficult in mungbean than that obtained in other grain legumes [14]. Notably, recovery of the regenerated plants significantly depends on the development of viable roots in vitro. The subsequent recovery of the transformed shoots has always been a great hurdle due to the recalcitrant nature of mungbean explants towards *in vitro* root generation. This ultimately greatly reduces the transformation efficiency in mungbean [17], making the process expensive, labour-intensive, and time-consuming, and ultimately hindering the widespread adoption of genetic modification in mungbean as a method of genetic improvement. The establishment of robust root systems in potential transformants is sometimes challenging due to extended exposure to high antibiotic concentrations throughout the selection process. Mungbean transformants maintained under prolonged antibiotic treatment yielded the same outcomes in a different set of experiments. In these conditions, micrografting might be a solution to the issue of successful root formation from *in vitro*-grown shoots of mungbean.

Micrografting is performed by grafting a meristem tip, a shoot tip, or a newly developed shoot onto a decapitated rootstock, aseptically. This technique has several benefits, including the eradication of viruses, year-round plant production, the creation of specialized genotypic combinations to boost plant productivity, and the development of genotypic combinations to augment plant yield and extend the ecological limits of a particular plant species [18]. The investigation of the histological characteristics of graft unions has also been made easier by micrografting [19]. The micrografting approach has not been used in root generation in mungbean. Therefore, successful regeneration and micropropagation of mungbean would be a promising and efficient approach.

To develop *in vitro* micro propagated plants, the problems addressed with *in vitro* root production and micrografting technique followed the possible solution [20-22]. Successful micrografting has been observed in various plants, such as cotton, fruit trees, species of woody plants, and a Leguminosae family member closely related to lentils and chickpeas [23-26]. There are no such reports discussing the use of micrografting to address issues with the growth of full plantlets in the case of Mungbean cultivars. Therefore, to successfully recover plants from *in vitro* regenerated shoots the techniques of micrografting were utilized. Thus, this study aimed to create an effective grafting method for Mungbean to help *in vitro* regeneration of complete plants.

MATERIALS AND METHODS

Plant seed collection and processing

The present study utilized mungbean [*Vigna radiata* L. (Wilczek)] seeds of the BARI Mung-3 variety, sourced from the Bangladesh Agricultural Research Institute (BARI) at Joydebpur, Gazipur. Cotyledonary node (CN) explants were obtained from 3-day-old seedlings grown *in vitro*. The explants were generated by detaching 4.0 mm sections of both the epicotyl and hypocotyl.

Media and growth condition

For *in-vitro* regeneration, MSB5 media BAP and IBA hormone were used in this present study. The composition of the MSB5 medium is followed by Gamborg [27]. The cultures were maintained at a temperature of 25 ±1°C with a 16/8-hour (light/dark) photoperiod using a cool white, fluorescent light (3000 lux). A fluorescent microscope was utilized to capture an image of the process of graft union development.

In vitro micropropagation

To prepare CN explants, we sterilized healthy and uniform seeds by rinsing them with tap water and then washing them with 70% ethanol for 1 minute. To enhance the sterility of the seeds, a solution of 0.1% $HgCl_2$ (W/V) was applied for 10 minutes. This was followed by 4-5 cycles of rinsing with sterilized distilled water. The seeds that had been sterilized on the surface were germinated in a sterile manner on Petri plates containing half-strength MS medium and 0.8% agar. The germination process took place at a temperature of 25°C under dark conditions [28]. To induce shoot regeneration, the plant tissue samples were placed on MSB5 medium supplemented with different concentrations of BAP (0.0, 2.5, 5.0, 7.5, and 10.0 μM) [29]. In addition, the explants were primarily cultivated for 30 days, with sub-culturing occurring at intervals of 15 days on the medium indicated earlier.

Micrografting

Once the shoots had undergone enough growth and elongation, they were transferred to a rooting medium with half the strength of MSB5 medium. This medium was treated with different concentrations (1.0, 1.5, 2.0, and 2.5 μM) of IBA. For micrografting, scions consisting of shoots that were produced in vitro and were 28 days old were utilized. The rootstocks were created using seedlings that were grown in vitro for either 7 or 14 days. To prepare the rootstocks, the shoot tip, axillary buds, and cotyledons were removed from the seedlings using a surgical blade. Upon removing the upper sections, the uppermost cut surface of the shoot was longitudinally split open to a depth of 50 mm. To achieve these objectives shoots measuring 1.5 - 3.0 cm in length were carefully removed as scions. A cut in the shape of a "V" was then made at the base of the scion. The grafting process involved gently inserting the scion into the vertical split of the rootstock, aligning a portion of the cambium layers of both the scion and rootstock (Figure 1). Following the insertion of the scion, the grafted area was securely sealed with masking tape and then enveloped in a translucent polythene bag to ensure optimal humidity levels. The process of strengthening micrografted plantlets was carried out at optimal room temperature conditions at 25±1°C, with a day length of 16 hours, until the graft union was successfully formed. During the initial 14-day period, the bags were systematically punctured to facilitate the exchange of air for the purpose of plant acclimation.

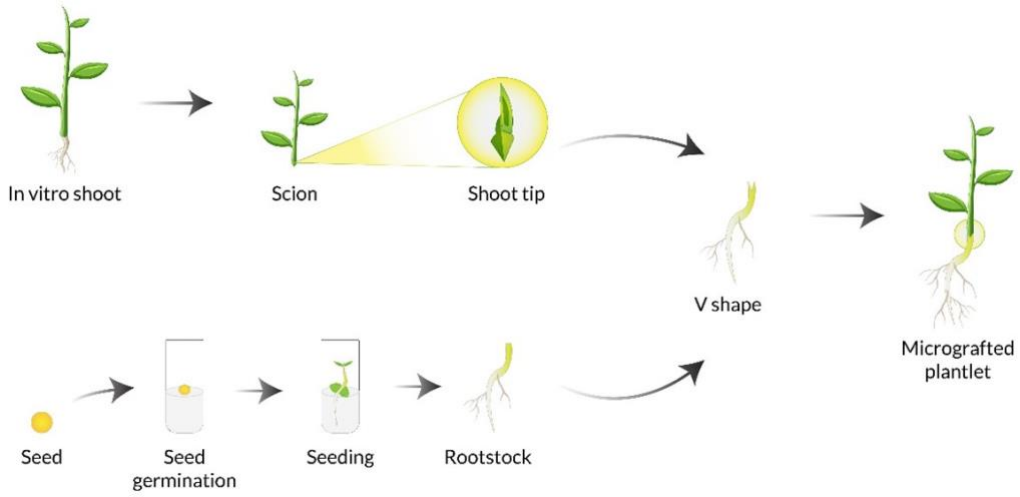

Figure 1. Diagram illustrating the procedure of micrografting. Scions are derived from shoots that have been regenerated in vitro and then sliced into 'V' shapes. A seedling that has been grown in a laboratory setting and had its top part removed at the point where the stem emerges is shown to have the rootstock cut in a similar 'V' shape. The process of grafting shoot tips onto the rootstock is achieved through top grafting using masking tape. A micrografted plant has been successfully produced, and the circular marking marks the specific place where the micrograft has been performed.

Transplantation

Following the process of acclimatization, the plants that were successfully grafted were relocated to larger earthen pots within a net house, where they were exposed to a natural environment until the growth of flowers and pods. The survival rate of the grafted plants was assessed after 30 to 40 days by analyzing the data that had been collected. Moreover, anatomical studies were done to comprehend how graft union was established in the case of Mungbean by using a fluorescent microscope.

Statistical analysis

The experiment was repeated three times, with each treatment being replicated 20 times. The studies were conducted in the laboratory using a completely randomized design (CRD). The data were statistically examined using Duncan's multiple-range test. The data obtained from several metrics, such as the percentage of regeneration, the number of shoots per explant, and the percentage of root initiation, were computed using different statistical measures: percentage, average, and standard deviation, respectively.

RESULTS

Successful shoot regeneration of mungbean variety BARI Mung-3

Several tests were conducted using aseptically grown explants (CN) of the Mungbean variety BARI Mung-3 to accomplish shoot regeneration in vitro. In order to achieve this objective, we used MSB5 media supplemented with varying amounts of BAP and kinetin to induce the growth of shoots in vitro. We utilized cotyledonary node explants for this purpose, as shown in Figure 2a. The impact of varying quantities of BAP and kinetin on the process of shoot regeneration in the Mungbean variety was observed to be inconsistent, as indicated in Table 1. Upon the addition of 5.0 M BAP to MSB5 medium, almost 80% of cotyledonary node explants exhibited a positive response to shoot

induction. The BARI Mung-3 variety had the largest average number of shoots per explant, which was 5.36 ± 0.56 (80.08%) (Table 1).

Table 1. Investigation of the effects of varying doses of BAP and their kinetics on shoot regeneration from cotyledonary node explants of the BARI Mung 3 variety.

BAP and kinetin applied in MSB_5 medium		Responses of explants towards regeneration of shoots (%)	Days to shoot initiation	Number of shoots per explant (Mean ± SD)	Mean length (cm) of shoot after 8 weeks of culture
BAP (µM)	Kn (µM)				
0.0	0.0	39.93	18-20	2.07 ± 0.78	2.76
2.5	0.0	55.47	12-14	3.57 ± 0.50	2.96
5.0	0.0	80.08	10-12	5.36 ± 0.56	3.16
5.0	0.46	72.52	10-12	4.83 ± 1.39	3.21
5.0	1.39	77.33	12-14	5.1 ± 1.35	3.32
5.0	2.22	75.35	12-13	4.03 ± 0.85	3.41
7.5	0.0	68.24	11-13	4.22 ± 1.17	3.36
7.5	0.46	63.34	12-14	4.36 ± 1.12	3.41
7.5	1.39	66.52	13-15	4.3 ± 0.99	3.51
7.5	2.22	64.57	13-15	4.5 ± 1.13	3.61
10.0	0.0	49.23	12-15	3.83 ± 0.91	3.55

Production of efficient rooting of mungbean

It was recorded that on average 12 days were required for the initiation of shoot from cotyledonary node explants (Figure 2b), while the formation of multiple shoots occurred at least 30 days from the initiation of culture. Following this experiment, *in vitro* raised multiple shoots have been presented in Figure 2c. After the development of sufficient healthy shoots, they were cultured in different media for the induction of roots. To do this, 3-5 cm long shoots were cut off and cultured on half-strength MSB media with different IBA concentrations (Table 2). Viable and functional root development was not successful due to the small number of roots that emerged and were not elongated enough to facilitate successful transplantation in the soil. Besides, the survival rate of the *in vitro* rooted plantlets was extremely low. So, in this case, the overall performance of the *in vitro* root development was not satisfactory. Under these circumstances, micrografting techniques were employed to get around obstacles to proper root formation and to increase the survival of *in vitro* generated plantlets.

Table 2. The impact of varying concentrations of IBA on the development of roots from in vitro propagated shoots in the BARI mung-3 variety was investigated using a half-strength MSB_5 medium.

Media composition	No. of shoots inoculated for rooting	No. of shoots rooted	% of shoots produced roots	Days to initiate roots	Days required to get well-developed roots	Mean number of roots/shoots
1/2 MSB_5	30	5	16.66	20-22	35-40	5.2 ± 1.30
1/2 MSB_5 + 1.0 µM IBA	30	6	20.00	16-18	32-35	5.3 ± 1.21
1/2 MSB_5 + 1.5 µM IBA	30	6	20.00	18-20	30-32	6.0 ± 1.26
1/2 MSB_5 + 2.0 µM IBA	30	8	26.66	13-15	30-32	6.8 ± 0.71
1/2 MSB_5 + 2.5 µM IBA	30	7	23.33	20-22	35-37	5.0 ± 0.58

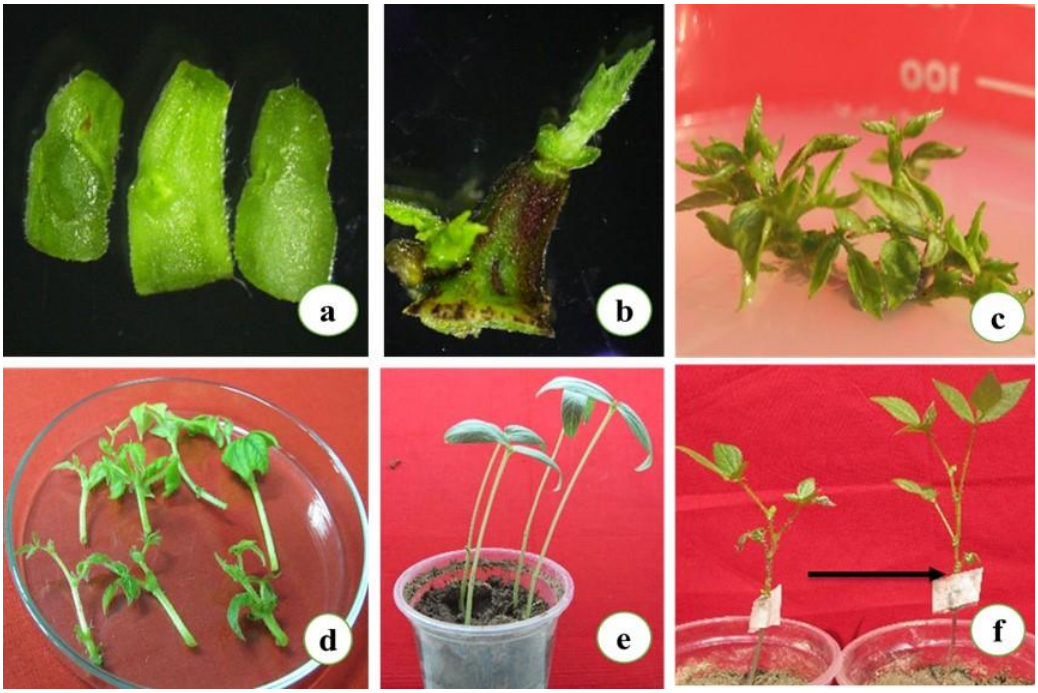

Figure 2. *In vitro* regeneration of shoots and stages of micrografting in BARI Mung 3 variety. a) Cotyledonary node (CN) explants excised from 3-day-old *in vitro* grown seedlings. b) Initiation of *in vitro* shoots from CN explant. c) Multiple shoots developing from CN explant on MSB_5 medium supplemented with 5.0 µM BAP. d) *In vitro,* raised shoots are used as scions for grafting. e) Seedlings prepared to use as rootstock for micrografting, and f) Successful micrografted plants after 33 days of grafting. Arrows indicate the grafted region covered by masking tape.

Scion length and rootstock age are vital factors for successful micrografting

Several factors were found to be associated with the development of a protocol for micrografting in Mungbean. It was recorded that scions (Figure 2d) having a length of 2.5 - 3.0 cm exhibited better response in establishing micrografts with a success rate of 55 % (Table 3). The second highest rate of grafting (48%) was obtained from the scions, which had a length of 1.5–2.0 cm. Identical observations regarding the role of scion length in graft formation were reported in the case of sandalwood plants [30]. It was observed that the 14-day-old rootstocks (Figure 2e) were more efficient than the 7-day-old rootstocks in achieving the desired micrografts (Table 3).

Table 3. The impact of scion length and rootstock age on the success rate of grafting.

Length of scion (cm)	Age of rootstock (days)	No. of grafts	Success of graft unions	% of successful grafts
1.5-2.0	7	20	7	35
2.5-3.0	7	20	8	40
1.5-2.0	14	20	9	40
2.5-3.0	14	20	11	55

Factors associated with decreased rate or failure of micrografting

The success rate of grafting decreased when the age of the rootstock exceeded 28 days. It was also noticed that the survival of the successful grafts was unaffected by the scion insertion and the extraction of the cotyledons from the rootstock. Despite the grafting method being less complicated and easy to perform, the chances of failure in grafting (63%) with the young rootstocks (7 days old) and smaller scions (1.5 - 2.0 cm) lengths. The displacement of the microscope has failed micrografting. The average success rate of grafting in mungbean plants was 51% (Figure 2f). After the establishment of a successful graft, the average success rate of transplantation was 81.66 (Table 4). The plantlets that were propagated grew very well and exhibited no morphological abnormalities. Consequently, Mungbean plants that had undergone micrografting had normal flowering and pod development (Figures 3a and 3b). The pods reached maturity and bore viable seeds (Figure 3c).

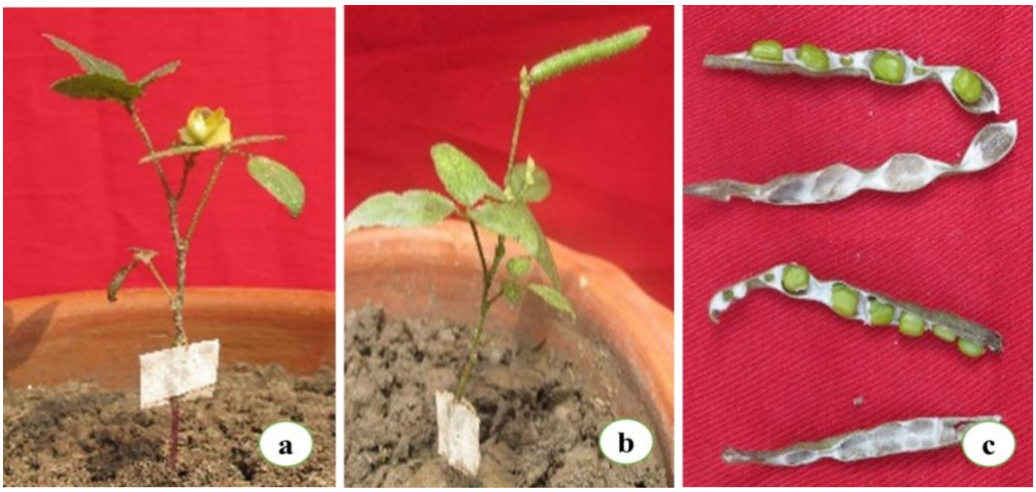

Figure 3. Development of flowers and pods on micrografted plants of BARI Mung 3 variety. a) Flower developed on a grafted plant indicating the successful establishment of a grafted plant. b) Pod formation on micrografted plants. c) Several healthy seeds were obtained from pods developed on the micrografted plant of Mungbean.

Table 4. The postoperative success rate of grafts and transplants following 30 days.

Lot Number	Number of grafting	Number of grafts produced	Rate of success (graft)	Successfully transplants	Rate of success (transplants)
Lot-1	23	11	48	8	72
Lot-2	24	12	50	10	83
Lot-3	20	11	55	10	90

To comprehend the nature of graft union anatomical studies were carried out particularly several transverse sections were done through the regions of graft formation and were observed under the microscope. To develop graft union formation between rootstock and scion the most

An important advancement is the establishment of vascular continuity across the interface zone. Successful grafting occurred only when there was a complete fusion of the xylem and phloem of the scion and rootstock. The integration of vascular bundles between the scion and rootstock was observed to be fully established 30 days following the grafting process. Callus production is a crucial phase for the establishment of a vascular bundle since it effectively connects the scion to the rootstock. Based on current

observations, cellular division initiates from the canter region of the union where the rootstock and scion are united. Various stages of graft union are presented in Figure 4a. A fluorescent micrograph of the transverse section of the stem showing the position of vascular tissue (Figure 4b). Fluorescent microscopic observation through the transverse section demonstrated the position of the vascular tissues of Mungbean shoot as well as the consequences graft union particularly the changes that occurred within the xylem and phloem. These observations indicated the establishment of the connectivity between the vascular tissues of the scion and rootstock (Figure 4c).

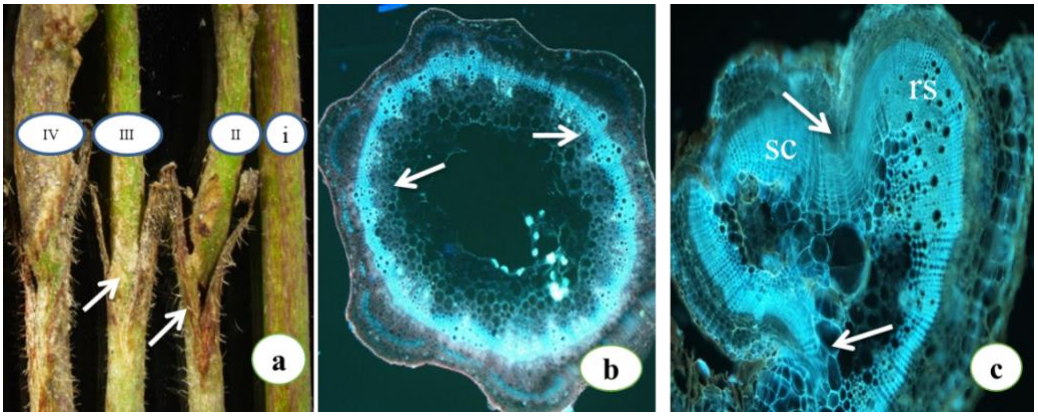

Figure 4. Graft union and anatomy of graft formation. a) Various stages of graft union, i. a control shoot, ii. & iii showing stages of establishment of graft, iv. fully establish a micrografted region. Arrows indicate a developing grafted region. b) A fluorescent micrograph of a transverse section of a control shoot showing the position of vascular tissues (arrows). c) A fluorescent micrograph shows the connected vascular tissues (arrows) of the scion (sc) and rootstock (rs).

DISCUSSION

Mungbean is a recalcitrant plant, presenting significant challenges in developing complete plantlets through micropropagation. One of the primary difficulties in micropropagation for some species is inducing adventitious root formation [31]. Under those circumstances, micrografting is an alternate method used in vitro to facilitate root growth and overcome challenges in rooting during the vegetative development of these species. Numerous genotypes of leguminous crop plants have reported experiencing such challenges in developing functional roots [32]. More particularly, several investigations have described issues with the development of *in vitro* roots from regenerated shoots in grain legumes like lentils, peas, and chickpeas [24, 33-36] as difficult. Additionally, it has been documented that in certain leguminous species, the prolonged presence of auxin in the rooting medium did not facilitate the typical rooting process from regenerated shoots [11]. While the use of full-strength MS media is common for in vitro rooting in various species, the high quantities of macronutrients in this medium can impede root initiation and lead to shoot necrosis after prolonged incubation [37, 38]. In the case of transgenic shoots, the *in vitro* regenerated shoots are cultured for a long period under antibiotic selection conditions and different BAP hormones that create a problem for proper root development. There are various reports that *in vitro* rooting in chickpeas has a negative correlation with added BAP concentration resulting in a 35% decrease in rooting [39, 40]. BAP negatively affected the *in vitro* rooting of Mungbean during this study (data not shown), and many other crops have shown comparable effects. Moreover, in many instances when phenolic compounds are released, the growth of tissue in culture stops, which prevents root initiation and development and ultimately

causes explant mortality. These phenolics easily oxidize, which results in tissue blackening and medium browning [41]. So micrografting of Mungbean is a good alternative to produce a viable complete plant through micropropagation.

Various factors affect this micro grafting technique. It was found that the length of the scion and the age of the rootstock employed impacted the rate of successfully grafted shoots during the current micrografting experiments. According to Nelson 2006 [42], in the case of *Acacia koa*, the grafting fails due to the narrow stem diameter of the rootstock and young stem tissue of the scion used for ensuring proper positioning of the microscion onto the rootstock is crucial for establishing adequate contact, which is important for the formation of the graft union. The success rate of rooting of normal regenerated shoots is 26.66 or lower when using selection pressure but after *in vitro* micrografting the success rate is more than 55%. The development of vascular cambium tissues of the rootstock and scion plants must be placed in contact with each other for a successful grafting to take place, in my study good vascular connectivity occurs in mungbean plants. Prior studies have documented the successful micrografting of shoots in several grain legumes and fruit crops e.g. citrus [26], lentil [24], *Pisum sativum* [34], chickpea [35], horticultural crops [18], and woody plant species [43].

CONCLUSION

The present findings suggest that micrografting offers a robust solution to the rooting challenges in mungbean plants with successful root regeneration, providing a practical approach for plant tissue culture with vegetative propagation-based trait improvement projects. This study suggests the efficient plant tissue culture approach as well as the efficient plant regeneration technique of mungbean plants. This study could be useful to legume breeders or farmers for improving mungbean and other legume species using breeding programs.

AUTHOR CONTRIBUTIONS

SKB conducted the investigation, wrote the original manuscript, and created the figures. MMH conducted formal analysis and data generation, with references included. Revisions and evaluation by MAR. Conceptualization, draft editing, modification, and supervision were performed by RHS and MNI. All authors have reviewed and consented to the final version of the manuscript that has been published.

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CONFLICTS OF INTEREST

There is no conflict of interest among the authors.

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