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Study on Highly Efficient Direct Shoot and Root Organogenesis from Cotyledon and *In vitro* Derived Root and Leaf of *Citrus jambhiri* Lush.

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***Abstract*:** A protocol was developed for the direct regeneration of plantlets from *C. jambhiri* leaf explants that had undergone *in vitro* regeneration. The goal of the current investigation was to determine how well *C. jambhiri* seed cotyledons might be directly used to regenerate shoots. The cotyledons enlarged as the seeds initiated its germination.. In MS medium supplemented with casein hydrolysate, the highest response to cotyledon expansion was observed. The number of enlarged cotyledons cultured by the medium treated with 50 mg L^{-1} of casein hydrolysate was highest (96%) and was followed by 100 mg L^{-1} (84%) and 200 mg L^{-1} of casein hydrolysate (78%). On the synthetic medium supplemented with 50 mg L^{-1} of casein hydrolysate (1.628 g/cotyledon), 100 mg L^{-1} of casein hydrolysate (1.148 g/cotyledon), and 200 mg L^{-1} of casein hydrolysate (1.050 g/cotyledon), the largest cotyledons by weight were produced. When the seeds were inoculated on MS media supplemented with 1.0 mg L^{-1} of IAA and 1.0 mg L^{-1} of IBA, direct regeneration from the roots of germinating seeds was observed in *C. jambhiri.* On MS media supplemented with various combinations and quantities of plant growth regulators, leaves excised from axenic shoot cultures were utilised to stimulate organogenesis. Adding 0.50 mg L^{-1} of dicamba to the medium indicated minimum percentage of leaf callus. As the leaf gradually dried up, the callus began to regenerate on the callus induction medium. Depending on the particular Citrus species, the quality of the germplasm, and the tissue culture practitioner's experience, direct organogenesis results might vary. To obtain promising results, factors including sterilisation, culture medium composition, and ambient factors should be properly optimised.

Keywords: Direct regeneration, PGR's, Enlarged cotyledons, Casein hydrolysate, Conservation

Citrus is a genus of flowering trees and shrubs of the Rutaceae family, well known as the citrus fruit. It is one of the most frequently grown fruit tree crops in the world and is valued for its delectable flavour, brilliant colours, and invigorating perfume. Oranges, lemons, limes, grapefruits, and tangerines are just a few of the well-known fruits of the genus *Citrus*, each of which has unique qualities and culinary applications. One of the most significant commercial horticulture crops in the world is the citrus species, which is grown in more than 100 countries, mostly in the Mediterranean area (Pandey and Tamta 2016). It produces many, vivacious nucellar seedlings that are essentially identical to zygotic seedlings due to polyembryony in nature (Kour 2012). Genetic erosion occurs in citrus because it is nearly impossible to distinguish zygotic seedlings from nucellar seedlings *in vivo*. Although vegetative propagation has advanced to some level to address these issues, traditional techniques like as budding and grafting to maintain "true-to-type" are naturally linked to clonal degeneration, mostly occured by viral infections. Axillary vegetative portions used for long-term clonal replication produce genetic instability, disease vulnerability, and viral complexity (Chaturvedi et al 2001).

To reduce these constraints, a very effective *in vitro* procedure involving appropriate explants and cultures is required. In plant biotechnology, citrus *in vitro* micropropagation is a complex and successful method for growing citrus plants in carefully regulated lab environments. It entails the development of citrus plantlets from tiny explants, such as shoot tips, leaf segments, or meristematic tissues, in a culture medium that is nutrient-rich and fortified with growth regulators and other necessary ingredients. The *in vitro* method provides a useful and effective tool for the production of elite large-scale planting materials, genetic preservation, and citrus enhancement. Using a variety of tissues and organs, including shoot tips (Gereme et al 2018), nodal explants (Gereme et al 2018), epicotyl segments (Kour 2016, Sidhu) and cotyledons (Saini et al 2010) *in vitro* regeneration in *C. jambhiri* has been effectively shown. Additionally, explants taken from mature plants result in contamination and poor *in vitro* shoot and root development (Tallo et al 2013). In order to reduce contamination and get "true-to-type" plants, the commercial micropropagation methodology focused on axillary explants selection from in vitro grown seedlings (Shenoy and Vasil 1992). Moreover,

compared to indirect regeneration via the intermediary process of callusing, direct shoot organogenesis offers reduced somaclonal variability among the regenerants (Savita et al 2012). Citrus rootstocks and root explants are among the explants that are best at withstanding biotic stressors, including viral complex. Although there aren't many literatures on Citrus shoot induction from root segments (Saini et al 2010), there aren't any reports on the possibility of entire root explants for *Citrus jambhiri* direct organogenesis.

Direct regeneration of plantlets from somatic tissues is crucial because it results in plantlets that are true to type. Indirect regeneration of plantlets by callus induction offers a good deal of somaclonal diversity. Therefore, mass-multiplication of true-to-type plant propagules may benefit from direct regeneration of plantlets from explant. Numerous studies have been conducted on citrus species' direct regeneration from nodal segments and shoot tips (Taye et al 2018, Sharma and Roy 2020). There are, however, few study findings on direct regeneration from *Citrus* spp. cotyledon, leaf, and root. On the other hand, a large number of promising research results on indirect regeneration through callus initiation and plantlet regeneration are available (Badr-Elden 2017, Fatonah et al 2018, Sharma and Roy (2020). As a result, an effort was made in this project to standardise a procedure for the direct regeneration of plantlets from the cotyledon, root, and leaf of *Citrus jambhiri* Lush. This is the first account of direct organogenesis from *C. jambhiri* Lush's cotyledons, root, and shoot.

Vitamin supplements and growth media are essential for *in vitro* morphogenesis. One of the most used media for citrus micropropagation (Ali and Mirza 2006) is MS medium (Murashige and Skoog 1962). Moreover, the beginning of dormancy and the establishment of shoot and root architecture are significantly influenced by plant growth regulators (PGRs) and vitamins in the basal media (Nongmaithem et al 2020). For improved adaption (Kazan and Manners 2009) of *in vitro* plants, auxins such NAA, IAA, and indole-3-butyric acid (IBA) play a crucial role in root modulation. While cytokinins encourage the growth and multiplication of shoots, auxins aid in the creation of roots. It is possible to regulate the growth and development of the citrus plantlets by adjusting the concentration and ratio of these growth regulators in the culture media. Agar, carbohydrates, and amino acids are among the other ingredients used to help the formation and growth of the plantlets.

The explants grow into tiny plantlets under regulated environmental parameters, such as temperature, light intensity, and photoperiod. To preserve the plantlets' vigour and avoid nutrient depletion, they are sub-cultured onto new medium at intervals as they grow. Citrus plants may be propagated quickly as a result of this ability to produce many plantlets from a single explant. The plantlets are moved to *ex vitro* settings for acclimatisation once they have reached the proper size and are showing good development. This entails exposing the plants to ambient conditions, lowering humidity, and supplying the right amount of water and nutrients to the plants so that they may gradually adjust to the external environment. The plantlets can either be transplanted into the field or used for additional study and reproduction after a successful acclimatisation. Thus, this method allows for the quick multiplication of superior cultivars with desirable features as well as the preservation and trade of endangered and uncommon citrus types.

MATERIAL AND METHODS

Direct Regeneration from Different Explants of *Citrus jambhiri* Lush.

The current research was conducted at the Synthetic Seed Laboratory in the Department of Seed Science and Technology, Uttar Banga Krishi Viswavidyalaya, Pundibari, Cooch Behar 736165, West Bengal, India, from 2018 to 2020. It focused on the direct regeneration of shoots from various explants of *Citrus jambhiri* Lush.

Source of plant material: Matured fruits of *Citrus jambhiri* Lush. collected from Indian Council of Agricultural Research (ICAR) Central Plantation Crop Research Institute, Kahikuchi, Guwahati, Assam, India was used for collection of seeds.

Preparation of explants: To collect the seeds, mature *Citrus jambhiri* Lush. fruits were brought from the Indian Council of Agricultural Research's (ICAR) Central Plantation Crop Research Institute in Kahikuchi, Guwahati, Assam, India.

Media preparation and culture conditions: On MS medium, all *in vitro* research was conducted (Murashige and Skoog, 1962). Exact proportions of each component of the specific medium were combined to produce the MS media. The medium's final volume (1.00 L) was created by mixing Mili-Q water with 3% sucrose. Either 1N HCl (Himedia) or 1N NaOH (Himedia) was used to bring the pH of the medium to 5.8. 0.8% agar (Himedia) was used to solidify the medium. To melt the agar, the MS medium was heated on a hot plate magnetic stirrer while being agitated. The medium was then autoclaved at 15 psi for 15 minutes at 121ºC. The culture bottles were placed beneath the laminar air flow cabinet, and the medium was then poured into them. The treatments included the following: (1) MS + Casein-hydrolysate (50, 100, and 200 mg L^{-1}); (2) MS + IBA (1.0 mg L^{-1}); (3) MS + IAA (1.0 mg L^{-1} + IBA (1.0 mg L^{-1}); (4) MS + BAP (1.0 mg L^{-1}); and (5) MS + BAP (1.0 mg L^{-1}).

Direct Regeneration from Cotyledon

Inoculation of explant: A sharp knife was used to cut mature fruits of *C. jambhiri* Lush, and the seeds were manually removed. Seeds were surface sterilized for 10 minutes with 0.1% $HgCl_2$ (Himedia) then washed 3-5 times with Mili-Q water. Surface sterilized seeds were de-coated in a laminar air flow cabinet before being inoculated onto a basal MS medium that had been enhanced with the various quantities and mixtures of plant growth regulators mentioned above. Then, cultures were put in a culture environment at 25°C with 16/8 hours of light and dark phases. The level of light was kept at 2500 lux

Inoculation for direct regeneration from root: Similar to this, IAA and IBA were added in various concentrations and combinations to MS media before the surface sterilized seeds were infected, as shown in Table 2. The cultures were subsequently contaminated for six weeks at 25±2ºC with 16/8 h light and dark phases in the culture chamber.

Inoculation for direct regeneration from leaf: On MS basal medium, surface sterilized seeds were cultured to promote germination and the establishment of seedlings. The culture environment was set at 25±2ºC with 16/8 h of light and dark phases for the seeds in culture bottles. Leaf explant source material was taken from *in vitro* grown seedlings that were six weeks old. For leaf explants, healthy *in vitro* seedlings were chosen. With the aid of a scalpel, the lower sides of the leaves of *in vitro* grown seedlings were cut off, and those *in vitro* grown dissected leaves were inoculated on MS medium that had been enhanced with the various concentrations and combinations of plant growth regulators listed in Table 3. The cultures were once more incubated in the culture chamber at 25±2°C and alternated between light and dark phases for 16/8 hours.

RESULTS AND DISCUSSION

Direct regeneration from cotyledon: The objective of the experiment was to determine how efficiently cotyledons of *C. jambhiri* could be used to regenerate shoots directly. The cotyledons enlarged as the seeds started to germinate (Table 1, Fig. 5 A). It ranged from 26% to 96%. In MS medium supplemented with casein hydrolysate, the highest response to cotyledon enlargement was observed. The number of enlarged cotyledons generated by the medium treated with 50 mg L^{-1} of casein hydrolysate was highest (96%) and was followed by 100 mg L^{-1} (84%) and 200 mg L^{-1} of casein hydrolysate (78%). The weight of enlarged cotyledons ranged from 0.517 to 1.628 g/cotyledon on average (Table 1, Fig. 5A). On the synthetic medium supplemented with 50 mg L^{-1} of casein hydrolysate (1.628 g/cotyledon), 100 mg L^{-1} of casein hydrolysate (1.148 g/cotyledon), and 200 mg L^{-1} of

Table 1. Effect of various combinations and concentrations of plant growth regulators on direct regeneration from *C. jambhiri* cotyledons on MS medium

Treatments	No. of seeds produced enlarged cotyledons	Mean wt. of enlarged cotyledons (g)	No. of cotyledon produced multiple shoots	No. of cotyledon produced multiple shoots	No. of multiple shoots/ cotyledon	
					Individual cotyledon	Mean
T1	48 (96%)	1.628[a]	3.0	1	32.0[a]	15.0[a]
				2	12.0[b]	
				3	1.0[e]	
T2	42 (84%)	1.148[b]	0.0	-	0.0[f]	0.0[c]
T3	39 (78%)	1.050[b]	0.0	-	0.0[f]	0.0[c]
T4	28 (56%)	0.517[d]	0.0	-	0.0[f]	0.0[c]
T5	32 (64%)	0.553[d]	0.0	-	0.0[f]	0.0[c]
T6	37 (74%)	0.815[c]	4.0	1	3.0[d]	3.0[b]
				2	1.0[e]	
				3	7.0[c]	
				4	1.0[e]	
T7	13 (26%)	0.682[e]	0.0	-	0.0[f]	0.00
Total	239	-	7.0	-	-	-
Range	13-48 (96-26%)	0.517-1.628	0.0-4.0	-	1.0-32.0	3.0-15.0
Mean	34.14 (68.29%)	0.76	3.50	-	8.14	9.0

T1: Casein-hydrolysate @ 50 mg L^{-1}; T2: Casein-hydrolysate @ 100 mg L^{-1}; T3: Casein-hydrolysate @ 200 mg L^{-1}; T4: IAA @ 1.0 mg L^{-1}; T5: IBA @ 1.0 mg L^{-1}; T6: IAA @ 1.0 mg L^{-1} + IBA @ 1.0 mg L^{-1}; T7: BAP @ 1.0 mg L^{-1}.
*Values bearing same letter in the column are not significantly different at $p = 0.05$ of LSD

casein hydrolysate (1.050 g/cotyledon), the largest cotyledons by weight were produced. When MS media was supplemented with 50 mg L^{-1} of casein-hydrolysate and 1.0 mg L^{-1} + 1.0 mg L^{-1} of IAA + IBA, direct regeneration from larger cotyledons were observed (Fig. 1A, B, and C and Fig. 2A, B, and C). Only seven cotyledons formed direct shoots (Table 1, Fig. 5B), three on medium supplemented with 50 mg L^{-1} of casein-hydrolysate and four on medium supplemented with 1.0 mg L^{-1} of IAA + 1.0 mg L^{-1} of IBA, indicating that the direct regeneration from cotyledons was random. After three weeks of inoculation on the enlarged cotyledon, the seeds that were inoculated on the MS medium enriched with 50 mg L^{-1} of casein hydrolysate had indicated many globular growths (Fig. 1A, B). Few of the globular-growing cotyledons regenerated into rootless plantlets. In contrary to plantlets that were directly regenerated from the cotyledonary axis (Fig. 1D), regenerated plantlets' leaves were abnormal (Fig. 1A and B). Contrarily, the cotyledons that enlarged on the medium supplemented with 1.0 mg L^{-1} of IBA and 1.0 mg L^{-1} of IAA formed noticeable globular callus-like structures with light green coloring (Fig. 2A). These spherical objects gradually developed into plantlets (Fig. 2B, C, and D). Compared to plantlets regenerated from the cotyledonary axis, those on 1.0 mg L^{-1} of IAA + 1.0 mg L^{-1} IBA supplemented media were normal. On a media containing the same plant growth regulators, the medium with the highest number of shoots (32 shoots/cotyledon; Fig. 1B; Fig. 5B; Table1) was observed. This was followed by a medium with 12 shoots/cotyledon (Fig. 2D, Table 1).

Direct regeneration from root: When the seeds were inoculated on MS media supplemented with 1.0 mg L^{-1} of IAA and 1.0 mg L^{-1} of IBA, direct regeneration from the roots of germinating seeds was seen in *C. jambhiri* (Fig. 3A, B, and C). On the contrary, seeds developed into radicles and plumules concurrently (Fig. 3A [1]). Without contacting the medium, the tap root of seeds helped germination increased by about 2.50 cm (Fig. 3A [2]). When it made direct contact with the medium, shoots and roots immediately began to regenerate (Fig. 3B, C). Only two roots displayed direct regeneration (Table 2). According to the recommendations of (Sharma and Roy 2020), the direct *in vitro* regenerated shoots were transferred to the hardening chamber before being eventually transplanted to the field.

Direct regeneration from leaf: Simple protocol was developed for the direct regeneration of plantlets from *C. jambhiri* leaf explants that had undergone *in vitro* regeneration. On MS media that had been supplemented with various combinations and concentrations of plant growth regulators, leaves that were removed from axenic shoot cultures were utilized to stimulate organogenesis (Table 3). Adding 0.50 mg L^{-1} of dicamba to the medium indicated a few number of callus to appear on the leaf (Fig. 6). The callus gradually showed symptoms of regeneration on the callus induction media as the leaf gradually dried out (Fig. 4B). On medium enriched with 50 mg L^{-1} of dicamba, only 9.75% of the leaf callus had shown this kind of regeneration. Because

Table 3. Effect of various combinations and concentrations of plant growth regulators on *C. jambhiri* leaf regeneration into direct shoots

Treatments	Callus induction (%)	Regeneration on callus induction medium
2,4-D 1.0 mg L^{-1}	78.57	-
2,4-D 2.0 mg L^{-1}	64.51	-
2,4-D 1.0 mg L^{-1} + NAA 0.50 mg L^{-1}	100.00	-
2,4-D 2.0 mg L^{-1} + NAA 0.50 mg L^{-1}	100.00	-
Picloram 0.50 mg L^{-1}	88.88	-
Picloram 1.0 mg L^{-1}	90.91	-
Dicamba 0.50 mg L^{-1}	84.00	9.75%
Dicamba 1.0 mg L^{-1}	76.92	-
TDZ 0.25 mg L^{-1}	96.87	-
TDZ 0.50 mg L^{-1}	100.00	-

Table 2. Effect of plant growth regulators on direct shoot regeneration from roots of *C. jambhiri*

Treatment		No. of seed inoculated	No. of seedling responded to direct regeneration from root	% of response	Average no. of shoots per inoculated shoot
IAA	IBA				
0.50	0.50	60	0.0	0.00	0.00
1.00	0.50	60	0.0	0.00	0.00
0.50	1.00	60	0.0	0.00	0.00
1.00	1.00	60	2.0	3.33	4.50
1.00	1.50	60	0.0	0.00	0.00
1.00	2.00	60	0.0	0.00	0.00
1.50	1.00	60	0.0	0.00	0.00
2.00	1.00	60	0.0	0.00	0.00

it guarantees the highest genetic consistency of the emerging plants, direct regeneration of plantlets is frequently used for *in vitro* mass-multiplication of citrus. The innovative result of this work is direct regeneration from *C. jambhiri* cotyledons. Numerous studies on the indirect regeneration of citrus species' cotyledon-derived callus (via callus induction and shoot regeneration) have been conducted (Waghmare and Pandhure 2015, Badr-Elden 2017, Fatonah et al 2018). On the other hand, no literatures were found on the direct regeneration of shoots from the cotyledons of citrus species,

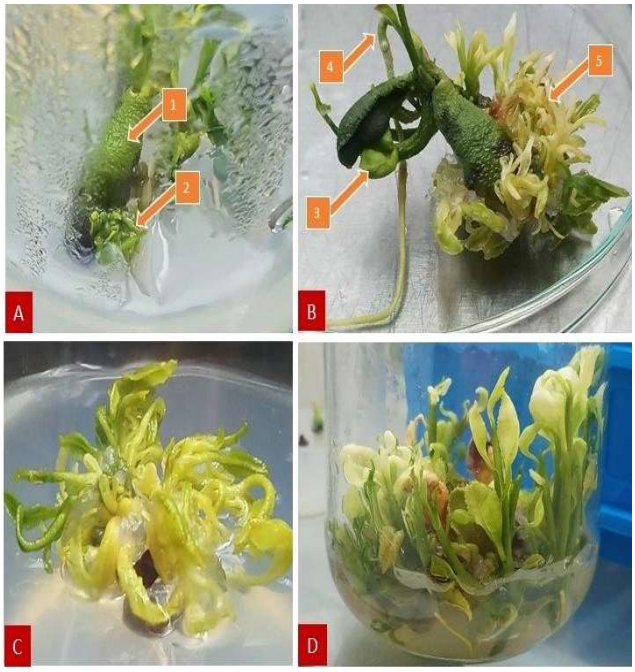

Fig. 1. When *C. jambhiri* cotyledons are inoculated on MS medium supplemented with 50 mg L^{-1} of casein-hydrolysate, direct regeneration results. A) Multiple buds on the cotyledon begin to form: A) Multiple buds that have sprouted, B) an enlarged cotyledon, C) an adult plantlet on the cotyledon: A multiple plantlet regeneration from the cotyledonary axis of germinating seeds when inoculated on MS medium supplemented with 50 mg L^{-1} of casein hydrolysate. 3] Plumule developed from the seed, 4] Radicle (root) developed from the seed, 5] Development of multiple plantlets; C) Developed multiple plantlets were separated from cotyledon and again cultured on MS basal medium

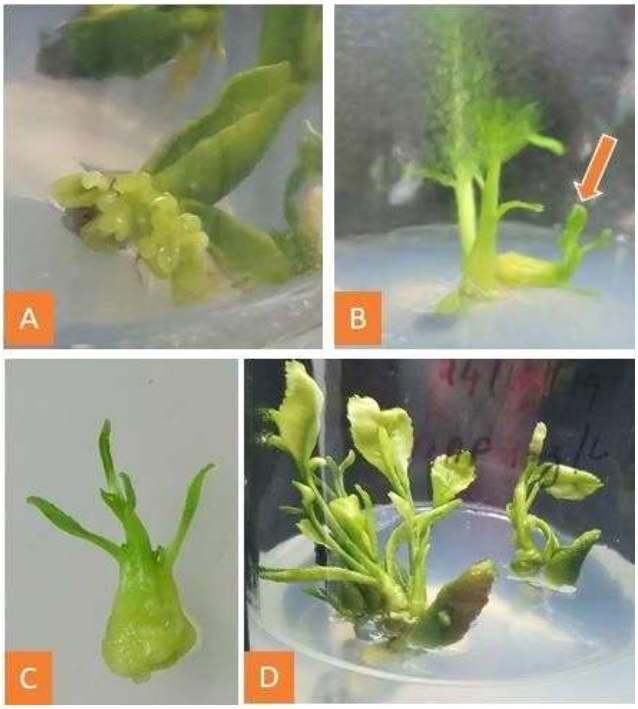

Fig. 2. Direct regeneration from *C. jambhiri* cotyledons. Direct embryogenesis on MS medium supplemented with 1 mg L^{-1} of IAA and 1 mg L^{-1} IBA in A; shoot regeneration on MS medium supplemented with 1 mg L^{-1} of IAA and 1 mg L^{-1} IBA in B; a magnified part of the regenerating shoot from the cotyledon in C; and multiple shoot regeneration on IAA and 1 mg L^{-1} IBA in D

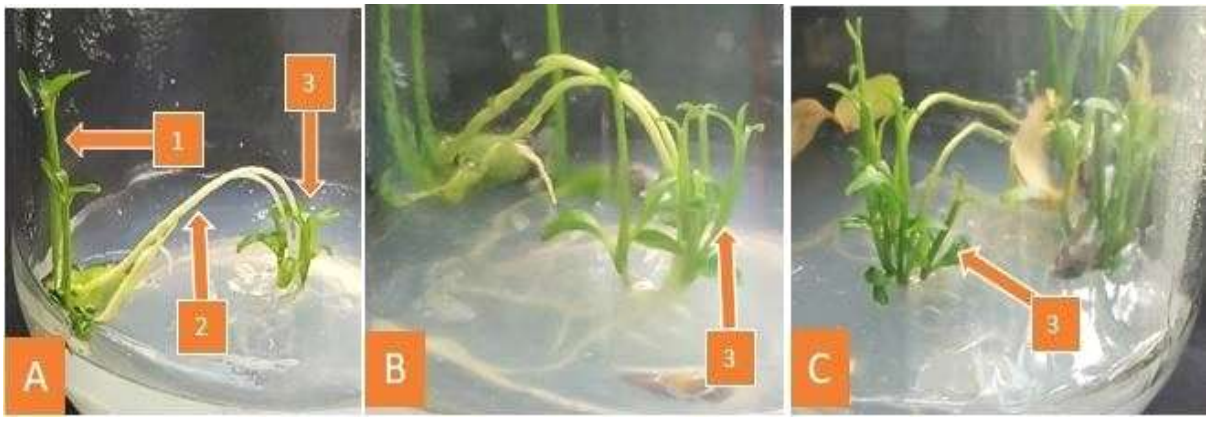

Fig. 3. Direct regrowth from *C. jambhiri* roots. Directly regenerated plantlets (A, B, and C): 1] Plumules from the germination of seeds, without touching the medium, the tap root of the germination seed expanded to a length of about 2.50 cm, and the shoots sprang directly from the root

according to a literature search conducted using Google Search. Numerous references are also available on direct shoot multiplication from nodal segments, shoot tips, cotyledonary nodes, axillary buds, and meristem culture in various citrus species (Taye et al 2018, Sharma and Roy 2020). When the MS medium was supplemented with 50 mg L^{-1} of casein hydrolysate and 1.0 mg L^{-1} of IAA combined with 1.0 mg L^{-1} of IBA, direct regeneration from cotyledons were achieved. When the MS medium was added with 50 mg L^{-1} of casein hydrolysate, a large number of plantlets regenerated from cotyledons; however, the health of the plantlets was poor, with the majority having semi-cylindrical leaves. However, some researchers have come to the conclusion that casein hydrolysate alone is more successful for plant tissue culture than the combination of the main amino acids. Casein hydrolysate reduces the glutamine deficiency when there is insufficient phosphorus for appropriate biosynthesis. As a result, it has been hypothesized that casein hydrolysates may include an unidentified growth-promoting component (George et al 2008). The majority of the plantlets produced on casein hydrolysate-added media were desiccated throughout the culture's later stages. Only a handful of the plantlets made it to the rooting media and survived. The plantlets that were grown on 1.0 mg L^{-1} of IAA plus 1.0 mg L^{-1} of IBA supplemented media, however, were healthy. After hardening, plantlets were rooted and transferred it in the soil. There are also relatively few reports on the direct regeneration of plantlets from roots. *C. aurantifolia* roots were continuously cultured for three years by Bhat et al (1992), who observed a low frequency of de novo shoot bud initiation in basal media throughout that time. Numerous studies have examined citrus species' roots-derived callus indirect regeneration (via callus induction and shoot regeneration) (Yaacob et al 2014). However, there are no current study data on direct regeneration from *C. jambhiri* roots. Our research on the direct regeneration of plantlets from roots is therefore novel, and this approach may be utilized to produce true-to-type propagules from roots.

Direct regeneration of plantlets from leaf segments is a viable method for mass-multiplications of citrus for maintaining genetic integrity. Direct organogenesis from *C. jambhiri* leaf explants is not yet observed. Explants grow buds or shoots when placed in a medium with a high cytokinin-to-auxin ratio (Schaller et al 2015). When the leaf explants of *C. limon* L. Burm cv. 'Primofiore' were cultivated on MS media supplemented with 3.5 ml L^{-1} of BAP, Kasprzyk-Pawelec et al (2015) also reported this form of direct organogenesis. In line with this, Hu et al (2017) findings revealed that cytokinin was the main cause of citrus shoot organogenesis. However, there are also instances on direct regeneration from plant species other than citrus that use leaves. An effective methodology for direct plantlet regeneration for the therapeutic plant *Aerva lanata* (L.) Juss. ex Schult. was standardized by Varutharaju et al (2014). Li et al (2013) developed an effective propagation and regeneration strategy in *Lysionotus serratus* via direct plantlet organogenesis from leaf explant. They discovered that Thidiazuron (TDZ) or 6-benzyladenine (BA) in high concentrations were efficient for direct organogenesis. A procedure for the direct regeneration of plantlets from male *Pistacia vera* L. cv. 'Atl' leaf explants was developed by Tilkat et al (2009). On a Murashige and Skoog (1962) medium with Gamborg vitamins added and various combinations and concentrations of BAP and IAA, leaves excised from axenic shoot cultures of pistachio were utilized to stimulate organogenesis. *Drosera rotundifolia* L. regenerated direct shoot as reported by Bobatk et al (1995) in either MS medium

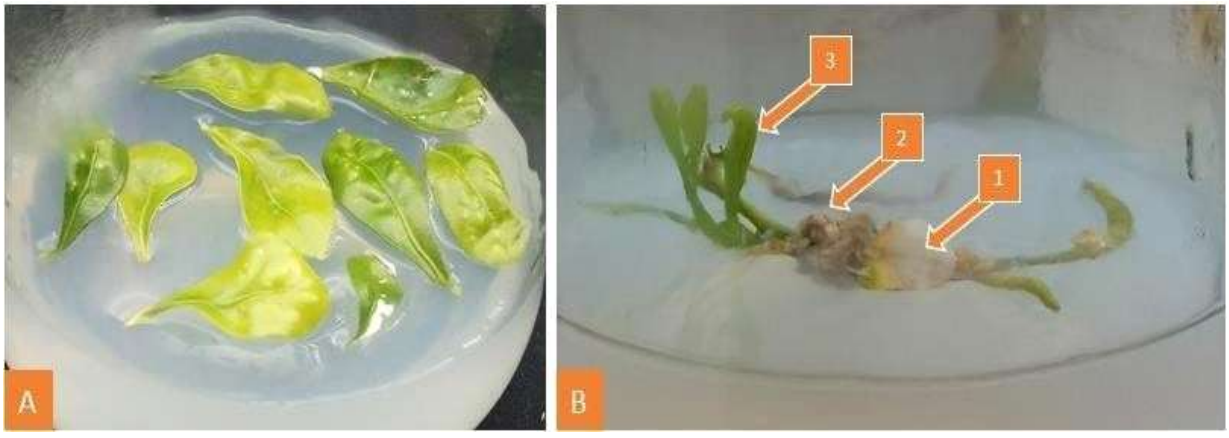

Fig. 4. Plantlets that are directly regenerated from leaves. A) *C. jambhiri* leaves that had been inoculated on MS media supplemented with 0.5 mg L^{-1} of dicamba; B) plantlets that had been regenerated from leaves: Following callus initiation, an inoculated leaf dries up, a tiny callus forms on the leaf, and a plantlet regenerates from the leaf callus on the callus induction medium

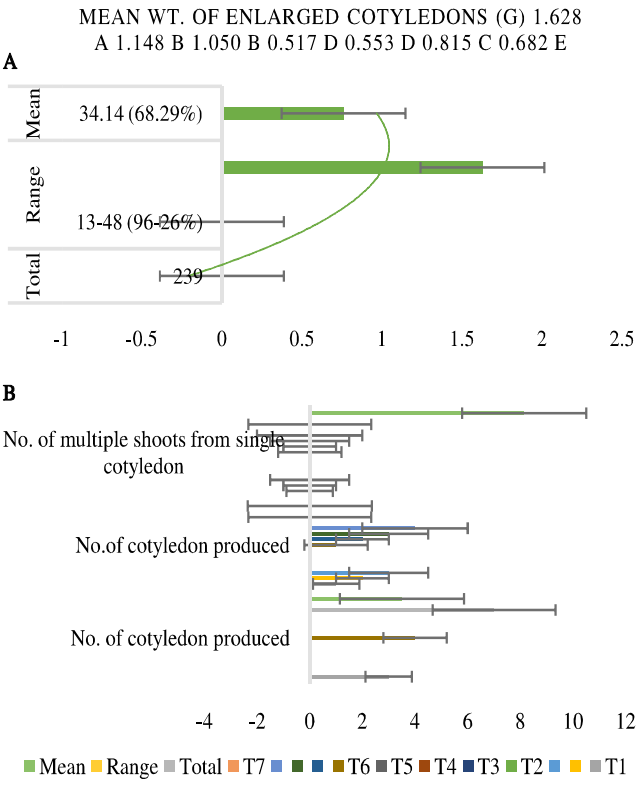

Fig. 5 (A-B). Influence of different combinations and concentrations of PGR's on number of seeds that produced enlarged cotyledons and its mean weight, number of cotyledons produced and number of multiple shoots emerged from a single cotyledon of *C. jambhiri* Lush

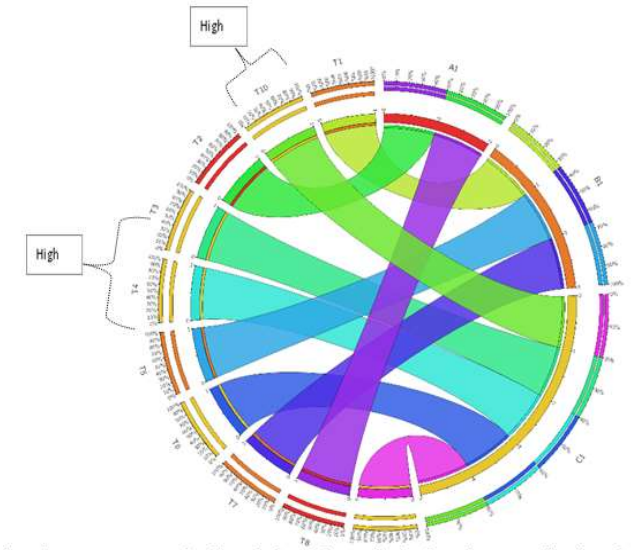

Fig. 6. Circos plot representing the percentage of callus derived from the leaf explants. Indicating that the maximum callus was obtained from the leaf segments treated with 2,4D (1.0+2.0) mg L^{-1} in conjunction with NAA (0.50) mgL^{-1} and TDZ (0.50) mg L^{-1}

supplemented with 10^{-8M} NAA or MS medium used as a basal medium. The ability of leaf tissue to regenerate was greatly improved by liquid culture media. Histological and scanning electron microscopy analyses confirmed their conclusions about direct shoot organogenesis, and it was shown that direct plant regeneration lacked intermediate callus development.

CONCLUSION

It is common practice to use *C. jambhiri* (Rough lemon) as the rootstock for planted citrus species. This report describes the direct regeneration of *C. jambhiri* plantlets from cotyledons, roots, and leaves. On a medium enriched with 50 mg L^{-1} of casein hydrolysate, 96% of the cotyledons grew larger. Few of the larger cotyledons regenerated branches directly. There might be a maximum of 32 shoots per responding cotyledon. In contrast, the plantlets' health was poor and appeared as semi-circular leaves. The majority of them died on rooting medium or dried out on maintenance medium. Healthy plantlets that had been regenerated on media were treated with IAA and IBA together with IBA established on maintenance medium and rooted on rooting medium. Additionally, leaf on MS medium supplemented with 0.50 mg L^{-1} of dicamba was successfully regenerated directly. This is the first study on the direct, extremely effective regeneration of shoot and root tissue from in vitro leaf and root growth. According to our research, plantlets from various *C. jambhiri* explants may be directly regenerated using tissue culture protocols in order to produce true-to-type plant propagules. Thus, citrus *in vitro* micropropagation is a useful technology for growing, preserving, and improving citrus plants. Citrus plants may be multiplied effectively and under control, supplying a steady stream of uniform, healthy plant material for breeding, research, and commercial orchards.

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