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In vitro plant regeneration in rough lemon (*Citrus jambhiri* L.)

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ABSTRACT

The present study was conducted to optimize the protocol for plant regeneration from stem, leaf, and root explants of rough lemon (*Citrus jambhiri* L). Explants from *in vitro* grown seedling of *C. jambhiri* were cultured on MS medium supplemented with various concentration of α-Naphthaleneacetic acid (NAA), 2,4-Dichlorophenoxy acetic acid (2,4-D) and 6-Benzylaminopurine (BA) for callus and shoot initiation. MS medium fortified with various concentrations of NAA were used for root formation. The range of callus initiation from stem explants of *C. jambhiri* was 13.33% to 80%, whereas 13.33% to 56.66% from leaf explants showed; and 6.66% to 36.66% from root explants. The frequency of shoot regeneration ranged from 13.33 to 70% from 15 days old callus. The highest frequency of callus initiation and shoot regeneration was observed in MS media supplemented with 1 mg L^{-1} 2,4-D and 0.5 mg L^{-1} BA; and MS media supplemented with 0.5 mg L^{-1} NAA and 3 mg L^{-1} BA, respectively. Rooting frequency ranged from 6.66% to 96.66% in the regenerated shoots. The acclimatized plants transferred to field condition survived at 100% frequency. MS media supplemented with 1 mg L^{-1} 2,4-D and 0.5 mg L^{-1} BA is the proper medium for high frequency (80%) callus induction in *C. jambhiri* using stem explant. MS media supplemented with 0.5 mg L^{-1} NAA and 3 mg L^{-1} BA and MS with 0.2 mg L^{-1} NAA are the best media for high frequency shoot regeneration (70%) and root initiation (96.66%), respectively.

Keywords: Citrus, explants, phytohormone, callus induction, shoot and root initiation

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1 Introduction

Citrus is a member of the Rutaceae family, which includes both edible and rootstock species as well as a few closely related genera, and it grows entirely in tropical and subtropical regions of the world (Taye et al., 2018). Globally, citrus fruits are grown over an area of 11.42 million ha with 179.0 million tons production (Singh et al., 2021). The nutritional and health benefits of citrus are well-documented (Altaf et al., 2008). Citrus and citrus products are ample sources of vitamins, minerals and dietary fibre that obligate normal growth and development. Citrus production in Bangladesh has been gradually increasing over the years, and the production of citrus fruit was 165.327 tons in 2019 (Roy and Sultana, 2021). It is grown by smallholders and commercial farmers, especially in the upland in the hilly areas of Sylhet, Chittagong and the Chittagong Hill Tracts. Among the citrus species, rough lemon (*C. jambhiri* L.) is a citrus hybrid associated with the citron, with the traits same as rangpur or mandarin orange. It is well adapted to warm-humid areas with deep sandy soils and shows resistance to viruses. In addition to the richest source of vitamin C and minerals, *C. jambhiri* is one of the most significant rootstocks for lemons, oranges, mandarins, grapefruits and kinnows (Savita et al., 2010). Trees grafted with this rootstock grow rapidly, remain productive for a longer time and produce excellent quality fruit in warm-humid areas with a deep sandy soils environment (Vij and Kuma, 1990; Beloualy, 1991; Savita et al., 2010). Citrus production by conventional methods is confined to particular season and accessibility of plant material (Usman et al., 2005).

The production of new varieties of scion and root stocks are limited in the traditional genetic plant improvement (Carimi and Pasquale, 2003; Mukhtar et al., 2005). In addition, high heterozygosity, auto incompatibility and a long juvenile period etc. are the barriers faced by the researcher (Germanà et al., 2011). Besides these, citrus species can be infected with disease-causing fungi, viruses, bacteria, mycoplasma etc. Attack of pathogens can cause considerable reduction of the yields and the quality of plants; even sometimes it can be lethal to plant. While pathogens are nearly always transferred in plants through vegetative propagation, viral diseases occur in virtually all seed propagated as well as vegetative propagated crop species (Taye et al., 2018). Therefore, the exclusion of pathogens is highly desirable to maximize the yields and quality and serve the movement of materials across international boundaries. Furthermore, the availability of an efficient regeneration system is also a prerequisite for genetic improvement and genetic resource conservation of citrus species (Saini et al., 2010). Hence, the application of tissue culture technology seems crucial to increase the production and maintain the desirable quality of plants, including citrus, to maintain desirable production. Researchers attempted to develop *in vitro* regeneration protocol of different citrus species using different plant growth regulators, explant types, and culture conditions (Usman et al., 2005; Khan et al., 2009; Laskar et al., 2009; Sharma et al., 2009; Pérez-Tornero et al., 2009). However, little work has been carried out on the *in vitro* regeneration of *C. jambhiri*.

Therefore, the primary purpose of the present work was to develop an efficient, stable and reproducible regeneration method of *C. jambhiri*. The effects of different explants of *C. jambhiri* under different hormonal combinations were tested in the present research to find out the optimal combination for high frequency of callus initiation and shoot regeneration in *C. jambhiri*.

2 Materials and Methods

2.1 Preparation of plant materials

Fresh and healthy seeds and fruits of *C. jambhiri* were collected from Citrus Research Center, Jaintapur, Sylhet, Bangladesh. The seeds were de-hulled and sterilized by three rinses of sterile distilled water followed by washing with 70% ethanol (MERCK, Germany) for 5 min with continuous shaking and then washed with distilled water three times. After that, seeds were washed with 10% Clorox (Sodium hypochlorite, The Clorox Company, Oakland, USA) solution for 15 min with shaking and rinsed with distilled water three times. Then the seeds were transferred into culture vessels for germination on half strength MS media containing salts and vitamins (Duchefa Biochemic, the Netherlands) and 3% sucrose (Murashige and Skoog, 1962). Four sterilized seeds were placed in each culture vessel, incubated at 25±2 °C temperature while maintaining a 16-hour photoperiod by white fluorescent light (144W).

2.2 Explant preparation and culture

Ten segment of stems, leaf and root explants each were excised from 2-week-old *in vitro* grown seedlings, and were placed per culture vessels containing 50 mL callus initiation medium containing MS media supplemented with different concentrations of BA (0.5, 1 and 2 mg L^{-1}), NAA (0.5, 1 and 2 mg L^{-1}) and 2,4-D (0.5, 1 and 2 mg L^{-1}). Stem and root segments were cut into 0.5–1 cm pieces as explants while leaf explants were prepared by cutting leaves perpendicularly to midrib with size of 0.5–1 cm (Fig. 1). After calli attained a convenient size, five calli were cultured in each culture vessel containing freshly prepared shoot regeneration medium (MS media containing various concentrations of NAA, BA and 2,4-D). When the shoots grew about 2-3 cm in length, they were separated and cultured in culture vessels containing freshly prepared root induction medium comprising MS media supplemented with NAA (0.1, 0.2 and 0.5 mg L^{-1}) to develop the root. Only MS media was used as control media in the case of callus and root initiation. In each step, the cultured vessels were sealed with Parafilm and marked with a permanent marker to indicate each treatment and were incubated in the culture room. When the rooted plantlets became 2-3 cm in length with a sufficient root system, these were very carefully taken out, washed and then transplanted sterilized pot soil. After proper hardening, the plantlets were moved to the natural environment.

2.3 Collection and analysis of data

The experiment was designed in a Completely Randomized Design (CRD) with three replications. Data were recorded on the percentage of callus initiation, percentage of shoot regeneration and percentage of root formation and statistically analyzed to determine the significance of the experimental results. The mean and standard deviation for all treatments were enumerated by using MS Excel 2013. The significance and mean separation tests were performed by using 'R' statistical package (R Core Team, 2021).

3 Results

3.1 Callus initiation

From all the 22 combinations of BA, NAA and 2,4-D used for callus initiation, the best callus percentage was obtained on MS medium supplemented with 1

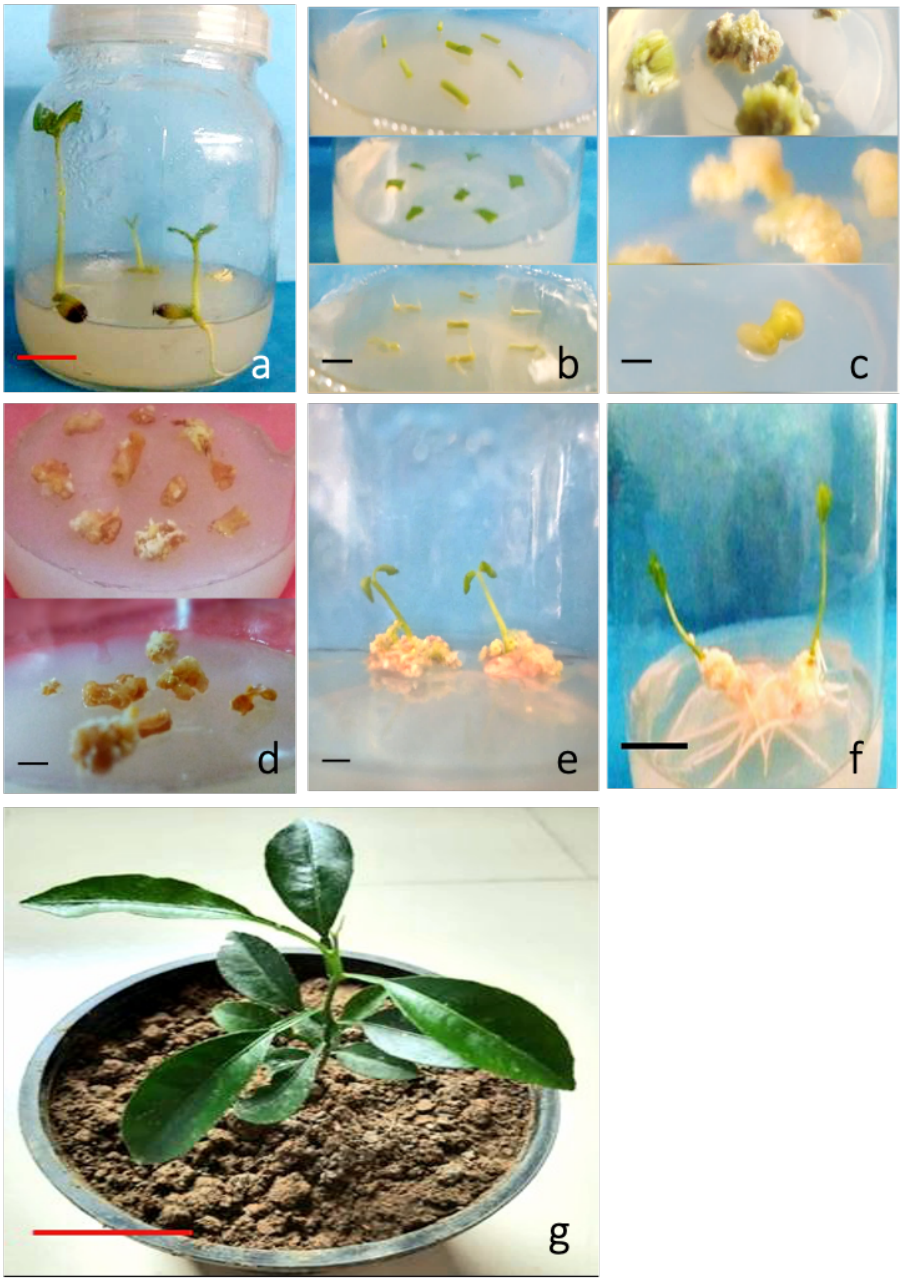

Figure 1. The *in vitro* regeneration process of *C. jambhiri* (a) two weeks old *in vitro* grown seedlings, (b) stem (upper panel), leaf (middle panel) and root (lower panel) explants on callus induction media, (c) calli produced from stem (upper panel), leaf (middle panel) and root (lower panel) explants at 15 days of culture, (d) necrotic callus from leaf (upper panel) and root (lower panel) explants after 2-3 subcultures, (e) regenerated shoots from calli, (f) *in vitro* rooting of regenerated shoots in root initiation media, (g) regenerated *C. jambhiri* plant in pot soil grown in natural conditions. Scale bars represent 5mm (b, c, d, e), 1 cm (a, f) and 2 cm (g)

mg L^{-1} 2,4-D and 0.5 mg L^{-1} BA (Fig. 2). Stem explants cultured on MS medium supplemented with 1 mg L^{-1} 2,4-D and 0.5 mg L^{-1} BA produced the highest 80% callus. Whereas, the lowest frequency of callus initiation (13.33%) was observed for stem explants in MS medium supplemented with 1 mg L^{-1} NAA and 2 mg L^{-1} BA. Stem explants did not produced any callus in MS, MS + 0.5 mg L^{-1} 2,4-D, MS + 1 mg L^{-1} 2,4-D and MS + 2 mg L^{-1} 2,4-D media combinations. In case of leaf explants the maximum callus induction response was 56.66% when MS medium supplemented with 1 mg L^{-1} 2,4-D and 0.5 mg L^{-1} BA and the minimal callusing response was 13.33% on MS medium supplemented with 0.5 mg L^{-1} NAA and 2 mg L^{-1} BA. Root explants showed the greatest 36.66% callus initiation frequency while MS medium supplemented with 1 mg L^{-1} 2,4-D and 0.5 mg L^{-1} BA and the lowest 0.00% in six media combinations *viz*. MS + 0.5 mg L^{-1} 2,4-D + 2mg L^{-1} BA, MS + 1 mg L^{-1} 2,4-D + 2 mg/BA, MS + 2 mg L^{-1} 2,4-D + 2 mg L^{-1} BA, MS + 0.5 mg L^{-1} NAA + 2 mg L^{-1} BA, MS + 1 mg L^{-1} NAA + 2 mg L^{-1} BA and MS + 2mg L^{-1} NAA + 2 mg L^{-1} BA.

3.2 Shoot regeneration

Various concentrations and combinations of BA, NAA and 2,4-D were tested (Table 1) to find out the best shoot regeneration response of *C. jambhiri*. Two weeks old calli were transferred in shoot regeneration media. Only stem explants were used for shoot regeneration because they showed better performance in callus production than leaf and root explants. Out of 18 combinations, the highest shoot formation frequency (70%) was found in MS medium supplemented with 0.5 mg L^{-1} NAA and 3 mg L^{-1} BA. The lowest shoot formation frequency (13.33%) was observed in MS medium supplemented with 1 mg L^{-1} 2,4-D and 1 mg L^{-1} BA.

3.3 Root formation

Finally, regenerated shoots were excised out and placed to rooting media MS medium supplemented with different concentrations of NAA (0, 0.1, 0.2 and 0.5 mg L^{-1}) (Fig. 3). Different root initiation frequency was observed in several concentrations of NAA. Plantlets showed the most significant frequency (96.66%) of rooting response when MS medium supplemented with 0.2 mg L^{-1} NAA and the lowest frequency of rooting (6.66%) in MS basal media (without NAA).

4 Discussion

Different types of explant were used by researchers for *in vitro* regeneration of citrus (Ali and Mirza, 2006; Savita et al., 2010). Here, the response of leaf, stem and root segments of *C. jambhiri* were tested under different hormonal combinations to find out the optimal combination for callus initiation. According to Sangma et al. (2020) combinations of PGRs were more effective than single concentrations of 2, 4-D, Kn and BAP for callus initiation. Stem explant was found as the most responsive among those three types of explants, which is consistent with previous studies (Ali and Mirza, 2006; Mohammad et al., 2015).

Media combinations were grouped into three classes: MS + 2,4-D, MS + 2,4-D + BA, MS + 2,4-D + NAA. After 5-6 days of culture, the explants became swollen and yellowish color callus formation started within 2 weeks. In the present study, MS + 1 mg L^{-1} 2,4-D + 0.5 mg L^{-1} BA was found as the best medium for callus initiation in the case of leaves, stem and root segment explants, and the response rate was 56.66%, 80% and 36.66%, respectively. Sangma et al. (2020) found 73.33% callus initiation while MS media supplemented with 0.5 mg L^{-1} 2,4-D and 0.25 mg L^{-1} Kn in dark condition. Khan et al. (2019) also reported the highest 86.7% callus formation in another species, *C. reticulata* on MS media containing 3.0 mg L^{-1} 2,4-D from leaf disc. More than 90% of callus formation was reported by Savita et al. (2010) in *C. jambhiri* using only 2-4,D. However, we observed maximum 37% callus initiation using only 2,4-D. This deviation may be happened due to the difference of explant age and habitat of the species. When explants were cultured in a hormone-free MS basal medium (control), they did not produce any callus, and explants died after a few days, which means growth regulators have a vital role in callus initiation of *C. jambhiri*. Root explant did not produce any callus in media combinations with higher BA (2 mg L^{-1}) concentrations in this study that might be the result of inhibitory effect of higher concentrations of BA on callus initiation.

Callus raised from leaf and root segments became necrotic after 2-3 subcultures. Identical results were obtained in previous studies (Mukhri and Yamaguchi, 1986; Savita et al., 2010; Yaacob et al., 2014). Therefore, healthy callus derived from the stem explants were used for shoot regeneration. Cell division and adventitious shoots differentiation from callus happen due to cytokinins (Pampanna, 2009). Apical dominance and release of lateral buds from dormancy are caused by these compounds while added to shoot initiation media (George et al., 2007). Higher cytokinin to auxin ratio and lower cytokinin to auxin ratio induces shoot and root respectively. In the present study, the highest shoot formation frequency (70%) was obtained in MS medium supplemented with 0.5 mg L^{-1} NAA and 3 mg L^{-1} BA followed by 60% shoot regeneration on MS medium supplemented with 2 mg L^{-1} BA and the lowest shoot formation frequency (13.33%) was obtained in MS medium supplemented with 1 mg L^{-1} 2,4-D and 1 mg L^{-1} BA.

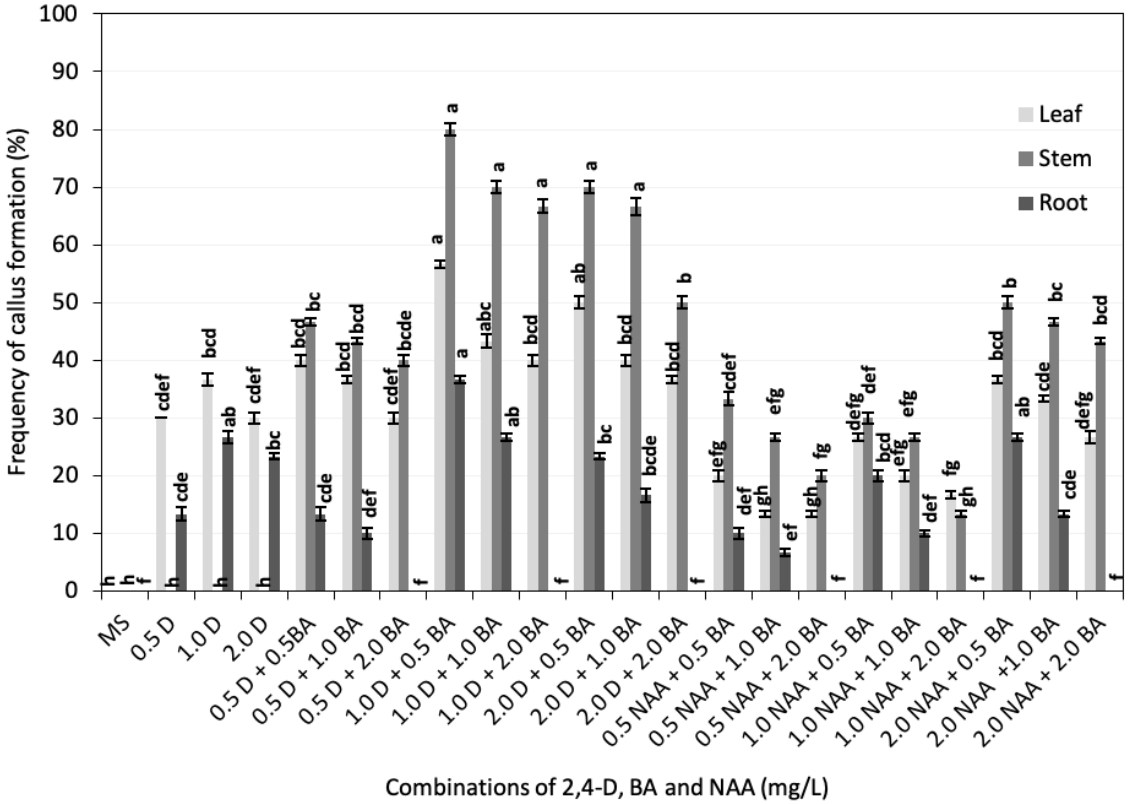

Figure 2. Frequency of callus initiation from stem, leaf and root explants of *C. jambhiri* on MS media supplemented with various concentrations of BA, NAA and 2,4-D at 15 days of culture. Data consists of three replications, and 10 explants were used for each replication. Bars represent SD of means. Mean ± SD followed by same letters are not significantly different at P = 0.05

Table 1. Frequency of shoot regeneration from 15 days old callus of *C. jambhiri* on MS media supplemented with various concentrations of plant growth regulators

Conc. of plant growth regulators (mg L^{-1})	Shoot regeneration frequency (%)
0.5 mg L^{-1} BA	23.33 ± 0.58 gh
1.0 mg L^{-1} BA	50 ± 1.0 bcd
2.0 mg L^{-1} BA	60 ± 1.0 ab
1.0 mg L^{-1} BA + 0.5 mg L^{-1} 2, 4-D	23.33 ± 0.58 gh
2.0 mg L^{-1} BA + 0.5 mg L^{-1} 2, 4-D	33.33 ± 0.58 efg
3.0 mg L^{-1} BA + 0.5 mg L^{-1} 2, 4-D	56.66 ± 0.58 abc
1.0 mg L^{-1} BA + 1.0 mg L^{-1} 2, 4-D	13.33 ± 0.58 h
2.0 mg L^{-1} BA + 1.0 mg L^{-1} 2, 4-D	30 ± 1.0 fg
3.0 mg L^{-1} BA + 1.0 mg L^{-1} 2, 4-D	43.33 ± 1.15 cdef
1.0 mg L^{-1} BA + 0.1 mg L^{-1} NAA	30 ± 1.0 fg
2.0 mg L^{-1} BA + 0.1 mg L^{-1} NAA	46.66 ± 0.58 bcde
3.0 mg L^{-1} BA + 0.1 mg L^{-1} NAA	60 ± 1.0 ab
1.0 mg L^{-1} BA + 0.5 mg L^{-1} NAA	50 ± 1.0 bcd
2.0 mg L^{-1} BA + 0.5 mg L^{-1} NAA	56.66 ± 0.58 abc
3.0 mg L^{-1} BA + 0.5 mg L^{-1} NAA	70 ± 1.0 a
1.0 mg L^{-1} BA + 1.0 mg L^{-1} NAA	26.66 ± 0.58 gh
2.0 mg L^{-1} BA + 1.0 mg L^{-1} NAA	36.66 ± 1.15 defg
3.0 mg L^{-1} BA + 1.0 mg L^{-1} NAA	53.33 ± 0.58 bc

Data consists of three replications and 10 explants were used for each replication. The mean values were compared by DMRT. Mean ± SD followed by same letters are not significantly different at P = 0.05

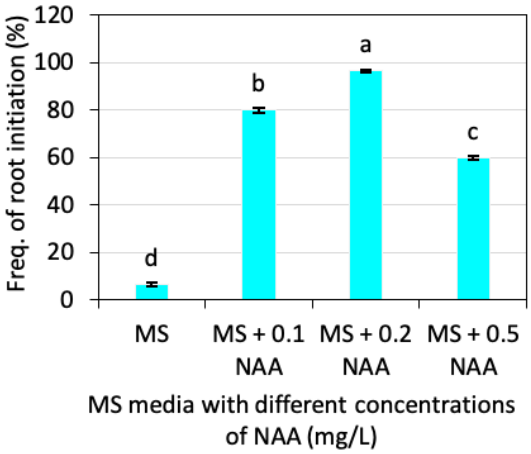

Figure 3. Impacts of MS and different NAA concentrations on the rooting of regenerated shoot of *C. jambhiri*. Data represents the mean of three replications where 10 regenerated shoots were used in each replication. Bar represents SD of means. Columns with different letters are significantly different (P = 0.05) from each other

Khan et al. (2019) reported higher shoot response (86.7% ± 3.35%) in MS medium supplemented with 3.0 mg L^{-1} BAP and 2 mg L^{-1} NAA in *C. reticulata*. Kaur (2018) reported that MS media supplemented with 0.5 mg L^{-1} NAA and 3.0 mg L^{-1} BAP and 1.0 mg L^{-1} kinetin had good regeneration potential for shoot regeneration. Taye et al. (2018) observed the highest shoot regeneration percentage in full-strength MS media supplemented with 0.1 mg L^{-1} of GA3 in nodal segments. Samila et al. (2015) reported the best combination for shoot induction media is MS media supplemented with 0.5 mg L^{-1} NAA and 0.5 mg L^{-1} BAP in *Citrus macroptera*. The highest shoot regeneration response 70% and 71.89% from callus cultured in MS medium fortified with 3 mg L^{-1} BA alone and in combination with 0.5 mg L^{-1} NAA, respectively, were observed in a couple of experiments (Ali and Mirza, 2006; Savita et al., 2010). Some study revealed that the combinations of BA and NAA are favorable for shoot regeneration from calli of different citrus species (Chaturvedi and Mitra, 1974; Beloualy, 1991). BA is reported as the best cytokinin in many studies for inducing organogenesis in citrus species (Carimi and Pasquale, 2003; Germanà et al., 2011). Many researchers suggested that use of BA alone gives better shoot regeneration of different citrus species (Raman et al., 1992; Costa et al., 2002). The combination of BA with NAA showed a better response (maximum 70%) than BA alone (maximum 60%) and BA with 2,4-D (maximum 56.66%). It is revealed that the shoot initiation frequency increased with the increase in BA concentration, which is consistent with Usman et al. (2005) and Goh et al. (1995).

As the lower cytokinin to auxin ratio induces ample roots with fewer shoots (Skoog and Miller, 1957), MS media fortified with only auxin (NAA) was used in this study for the root induction of regenerated shoots. Shoots developed from the stem callus were cultured in MS media and MS media added with various concentrations of NAA (0, 0.1, 0.2 and 0.5 mg L^{-1}) for root initiation. The highest (96.66%) rooting response was observed when plantlets were cultured on MS supplemented with 0.2 mg L^{-1} NAA and the lowest response, 6.66% was observed in only MS media. Sangma et al. (2020) reported the best rooting response in MS incorporated with 1.0 mg L^{-1} exhibited NAA in *C. indica*. Kaur (2018) reported highest rooting in MS supplemented with 1 mg L^{-1} NAA and 1.0 mg L^{-1} IBA. Sarker et al. (2015) observed best root induction (100%) on MS media fortified with 0.5 mg L^{-1} NAA in *Citrus aurantifolia*. Continuous addition of more NAA lowers the rooting frequency. Similar results were found by Savita et al. (2010) with MS media and by Devi et al. (2021) with MSN media.

5 Conclusion

Shoot, leaves and root explants of *C. jambhiri* can produce callus; however, the performance of stem explant produced the highest amount of calli among them. Maximum percentage of callus obtained with 1 mg L^{-1} 2,4-D and 0.5 mg L^{-1} BA. The best response of shoot initiation was observed in MS + 0.5 mg L^{-1} NAA + 3 mg L^{-1} BA medium. MS + 0.2 mg L^{-1} NAA gave the highest rooting of *C. jambhiri*. Thus, the MS media supplemented with these concentrations of NAA and BA could be used for the *in vitro* plantlets regeneration in rough lemon (*C. jambhiri*).

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Conflict of Interest

The authors declare that there is no conflict of interests regarding the publication of this paper.

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