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Research Article

In vitro callus induction and multiple shoot regeneration in rare Iranian endemic plant *Haplophyllum virgatum var.* virgatum

Somayeh Torabi[1], Farah Karimi[1*] and Khadijeh Razavi[2]
[1] Department of Biology, Faculty of Basic Sciences, Shahed University, Tehran, Iran
[2] National institute of Genetic Engineering and Biotechnology, Tehran, Iran
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Abstract

***Haplophyllum virgatum* var *virgatum* (Rutaceae) is a rare or narrow endemic plant, which grows only in one habitat (Geno-Hormozgan Province-Iran). Low distribution of this useful medicinal plant encouraged us to investigate the micropropagation conditions of the plant. In the present study, an efficient and reproducible protocol for multiple shoot induction of *Haplophyllum virgatum var.* virgatum was developed. Various explants (stem, leaf and root) were obtained from 40 day-old axenic seedlings and cultured on B5 medium supplemented with different concentrations of plant growth regulators (Kin, BA, NAA and IAA) to determine the suitable explants and media composition for callus production. BA and Kin were used for multiple shoot induction. Stem explants showed the best results in both callus induction and shoot regeneration. Combination of 0.1 mg L^{-1} Kin and 5 mg L^{-1} IAA produced the maximum callus fresh weight. BA at concentration of 2 mg L^{-1} was the best treatment for shoot induction and regeneration. Also, embryogenic callus was observed and the stages of globular and heart embryos were seen on callus culture in B5 medium supplemented with Kin (0.2 mg L^{-1}) + IAA (1 mg L^{-1}). In conclusion, stem explants and 2 mg L^{-1} BA regenerated maximum number of shoots.**

Keywords: Callus culture, *Haplophyllum virgatum*, Organ culture, Root regeneration, Shoot regeneration

Introduction

The genus *Haplophyllum* A. Juss. (Rutaceae) consists of 70 species found mainly in warm, temperate, and subtropical regions of the northern hemisphere of the Old World (Joharchi, 2008; Parhoodeh *et al*., 2011). The main center of diversity of *Haplophyllum* is Irano-Turanian region—in particular, Iran, Turkey, and Central Asia— that harbours 60% of the species diversity. This genus, with the local name of "Sodabi," reported in the flora of Iran by 30 species, of which 14 are endemic (Mozaffarian, 1996). *Haplophyllum* species are perennial herbs, sometimes low-shrubs, which grow mainly on sandy, stony, or rocky hill slopes in arid areas (Townsend, 1986). Morphologically, they can be broadly characterized by the presence of cymose and bracteates inflorescences with five sepals and creamy-white to bright yellow petals, ten stamens with free filaments expanded below and pubescent on the inner surface, three to five connate carpels, and five-lobed capsules (Townsend, 1986). It has been reported that this genus contains essential oils, alkaloids, fixed oils, coumarins, sterols, flavonoids as well as lignans (Al-Burtamani *et al*., 2005; Azadi and Khaef, 2015; Karimi *et al*., 2013; Mechehoud *et al*., 2014; Rasulova *et al*., 2015). Many species of the genus (*Haplophyllum* A. Juss.), are used in traditional medicine for the treatment of herpes, warts, stomachache, erysipelas, toothache, skin diseases (Bessonova, 1989) and in the treatment of testicular cancer (Ea *et al.*, 2008).

Endemic species grows naturally only in a single geographic area, the size of which could be either narrow or relatively large. Some species may be both rare and endemic if it lives in a narrow geographical area. These plants are narrow endemics, which exhibit as only few populations (Kruckeberg and Rabinowitz, 1985). Small populations, narrow geographical area and special habitat demands make these endemic species at the risk of extinction (Myers, 1988; Heywood and Watson, 1995). Direct or indirect organogenesis of endangered, endemic and rare plant species via tissue culture techniques has been used fruitfully to cope with the extinction problem without damaging wild populations (Bonnes *et al*., 1993; Carneiro *et al*., 1999). The several endemic species of *Haplophyllum* occur in small, distinct populations across their narrow range. These factors make many species and populations of this genus particularly vulnerable to extinction, a fact that has been recognized with the inclusion of nine species in the Red Data Book of Iran (Jalili and Jamzad, 1999). Plant tissue culture is a potentially useful technique for growing rare or threatened plants (Li *et al*., 2004; Emma *et al*., 2005). This technique produces a large number of diseased free plantlets at minimum time using only a minute amount of plant material. Also, using plant tissue culture techniques, seed dormancy and propagation difficulties are affectively

*Corresponding Author, Email: fkarimi@shahed.ac.ir

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removed. For conservation and economic uses, many endemics as well as endangered plant species have been successfully propagated in the past. Callus tissue is a good source of experimental tissue material from which whole plant can be regenerated by changing the nutrient and hormonal constituents in the culture medium. Reliable proliferation of callus and subsequent plant regeneration are important for massive plant propagation. However, few numbers of plant regeneration studies have been reported in *H. patavinum* (Puricelli *et al*., 2001), *H. tuberculatum* (Elmaghrabi *et al*., 2017; Abdallah *et al*., 2019) and *H. gilesii* (Kausar *et al*., 2019). This study describes the first successful *in vitro* regeneration system of *H. virgatum* as an Iranian endemic plant. The aim of this study was to investigate the effects of different concentrations of plant hormones and various types of explants on callus induction and shoot regeneration of endangered medicinal plant *H. virgatum* var. *virgatum* on in-vitro.

Material and methods

Plant material and seed culture: The seeds of *H. virgatum* var. *virgatum* were collected from Geno area in Hormozgan province of Iran (27˚ 27′ N latitude and 56˚ 18′ E longitudes at the altitude of 45 m). At first the seeds were washed under running tap water for 48 h, followed by sterilizing in 70% ethanol during 30 seconds and washing with distilled water, and then surface sterilized using 10% (v/v) commercial sodium hypochlorite (NaOCl) during 10 min and finally, washed three times with sterilized distilled water. Surface sterilized seeds cultured on solid B5 medium (Gamborg *et al*., 1968) and incubated at 25˚C, with 16 h photoperiod of light to produce aseptic seedlings. To study seed germination, 10 autoclaved jars used for each replication and six seeds sown in each of them. The experiment was repeated three times to study seed germination and seedling production.

Callus induction: Different explants including stem, root and leaf (0.5- 1 cm in diameter) obtained from *in vitro* grown up seedlings after 40 days, cultured for callus induction and growth optimization. B5 medium supplemented with different concentrations of kinetin (Kin) (0.1, 0.2 and 0.5 mg L^{-1}) and 6- benzyl adenine (BA) (0.1, 0.2 and 0.5 mg L^{-1}) in combination with α-naphthalene acetic acid (NAA) (1, 2 and 5 mg L^{-1}) and indole-3-acetic acid (IAA) (1, 2 and 5 mg L^{-1}) was incubated in darkness at 25˚C with 3 % sucrose. After two months, fresh weight of produced callus was investigated.

Shoot regeneration: After two months, derived calli were transferred to B5 medium supplemented with 1, 2 and 3 mg L^{-1} of BA and Kin for shoot induction. The number of regenerated shoots were recorded after two months. Cultures maintained at 25˚C for 16 h photoperiod of light.

Root regeneration: The regenerated shoots cut after elongation (2-3 cm) and transferred to ½ MS medium, in presence of NAA, IAA or indole-3-butyric acid (IBA) for root regeneration. They were cultured at 25ºC under 16 h photoperiod of light. Root number assessed after two months.

Statistical analysis: The data (3 replicates per treatment), were subjected to one-way analysis of variance (ANOVA) to assess treatment differences and interaction using SPSS software (version 16). Duncan's Multiple Range Test ($P < 0.5$) used for determination of significant differences between mean values.

Results and discussions

Seed germination and seedling production: Examination of seed germination on B5 medium showed that the seeds germinated after one week and germination rate was about 68% (Table 1). However, after one month, a few numbers of seeds grew and turned into seedlings (19.93%) (Figure 1, Table 1). According to these results, proliferation of *H. virgatum* by micropropagation is justifiable.

Callus induction: Callus induction, observed with different concentrations and combinations of PGRs using stem, root and leaf explants. Callus can be produced from a single differentiated cell, and many callus cells are totipotent, being able to regenerate the whole plant body. Therefore, the term callus includes cells with various degrees of differentiation (Feher, 2019). Callogenic response in different explants depends on morphological characteristics of explants, type and concentration of employed plant growth regulators. Callus produced from the wounded side of the explants and it is believed that its production is triggered by accumulation of hormones at wound sites (wound response) (Ahmad *et al.*, 2010) which is strengthen with exogenous usage of the growth regulators. Wounding was promoted callus formation in various parts of Arabidopsis seedlings. At least some aspects of wound induced callus formation were driven through the upregulation of cytokinin signaling (Iwase *et al*., 2011). The color of induced calli from explants in darkness, were cream or brown (Figure 2a, b). Both compact and friable callus textures were observed (Figure 2). Friable calli fall apart easily, and can used to generate cell suspension cultures. In our study, compact calli were generable from different explants and treatments but friable calli generated only in root explants and callus inducing treatments containing BA (0.1, 0.2 and 0.5 mg L^{-1}) + NAA (1, 2 and 5 mg L^{-1}) (Table 4). Also, embryogenic callus was observed and the stages of globular and heart embryos were seen on callus culture using B5 medium supplement with Kin (0.2 mg L^{-1}) + IAA (1 mg L^{-1}) (Figure 3). The formation of some calli uses intrinsic developmental programs, such as embryogenesis. It is likely that the hormonal and developmental pathways are interconnected at multiple levels (Ikeuchi *et al*., 2013). The role of auxins or its combination with cytokinins for callus induction and proliferation is well-documented (Roy and De, 1990). In our study, the combination of IAA and NAA as auxins and Kin and BA as cytokinins were used for

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Figure 1. *In vitro* seed germination and seedling growth of *H. virgatum* var. *virgatum* on B5 basal medium

Table 1. The percentage of seed germination and seedling production on B5 medium

	Seed Germination Percentage	Percentage of Plantlet Production
B5 medium	67.90 ± 1.05	19.93 ± 1.55

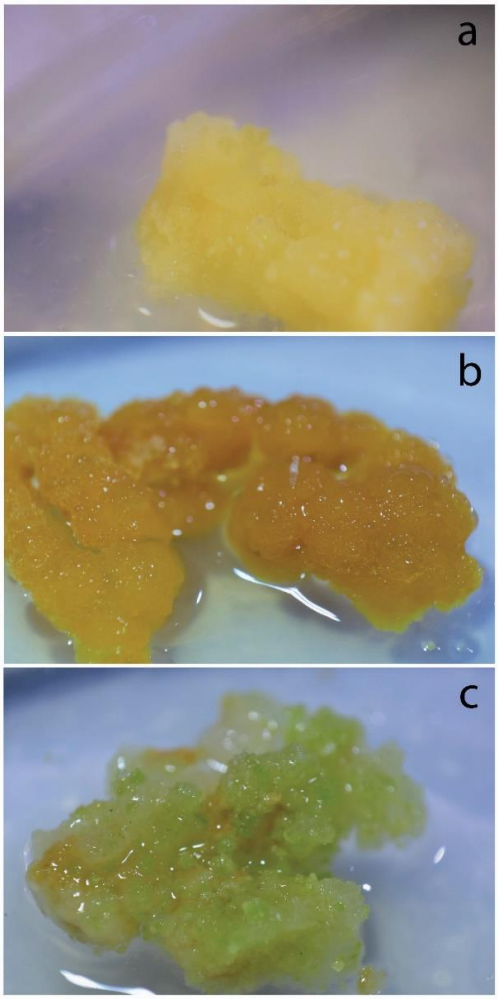

Figure 2. Types of induced calli. (a) Creamy compact callus from stem explant on B5 medium supplement with Kin (0.1 mg L^{-1}) + IAA (5 mg L^{-1}), (b) Brown compact callus from leaf explant on medium supplement with Kin (0.1 mg L^{-1}) + IAA (1 mg L^{-1}), (c) Friable callus from root explant on medium supplement with BA (0.1 mg L^{-1}) + NAA (2 mg L^{-1})

callus induction. Figures 4, 5 and 6, show the effect of different concentrations of plant growth regulators as well as their combination on callus fresh weight from different explants. The lowest callus fresh weight was observed in presence of 0.5 mg L^{-1} Kin in combination with 5 mg L^{-1} NAA (Figures 4 and 5). For root explants, the combination of BA and IAA did not produce any calli at all except for 0.1 mg L^{-1} BA and 1 mg L^{-1} IAA (Figure 6). Maximum callus fresh weight was produced in stem and root explants when they were cultured on

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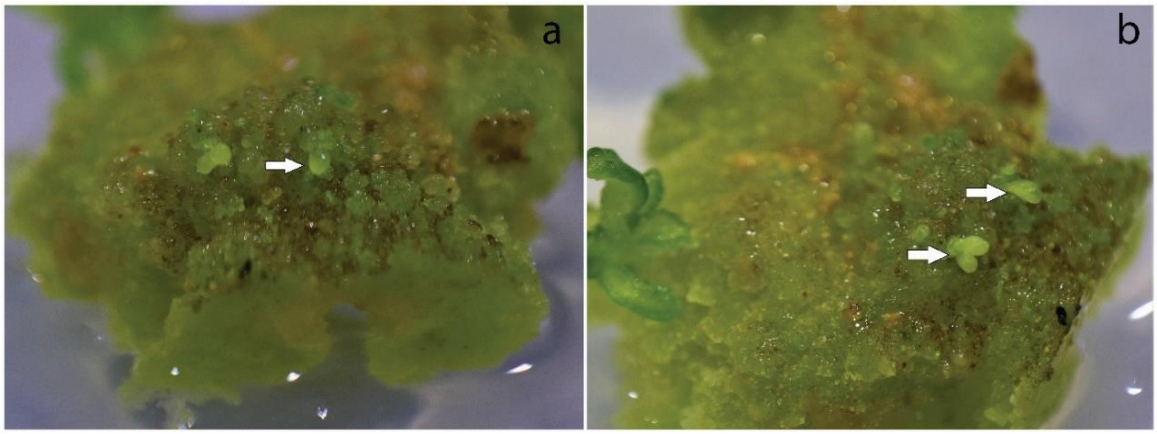

Figure 3. Somatic embryogenesis. (a) Globular and (b) Heart shape embryos, induction on callus culture using B5 medium supplement with Kin (0.2mg L^{-1}) + IAA (1mg L^{-1}).

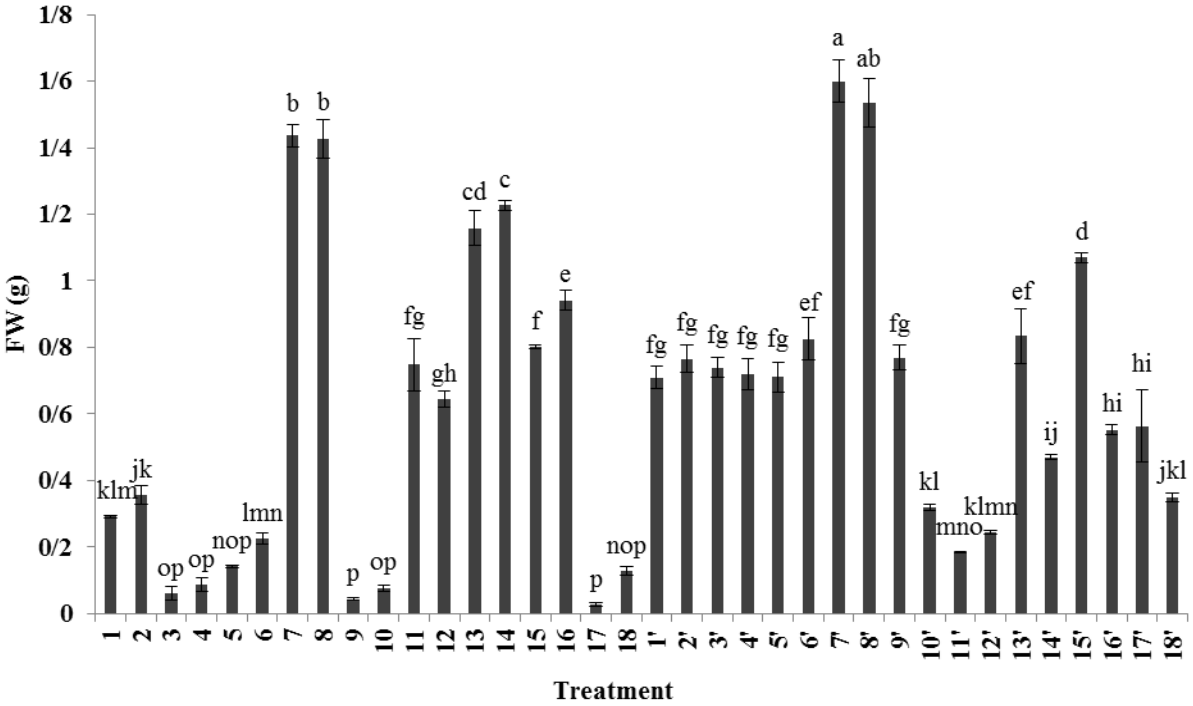

Figure 4. Callus fresh weights (g) under different employed hormonal treatments on stem explants. The vertical bars represent standard errors (SE). The mean values marked by the same letters are not significantly different at P<0.05 as determined by Duncan's multiple range test.

(1) Kin: 0.1 mg L^{-1}, NAA: 1 mg L^{-1}; (2) Kin: 0.2 mg L^{-1}, NAA: 1 mg L^{-1}; (3) Kin: 0.5 mg L^{-1}, NAA: 1 mg L^{-1}; (4) Kin: 0.1 mg L^{-1}, NAA: 2 mg L^{-1}; (5) Kin: 0.2 mg L^{-1}, NAA: 2 mg L^{-1}; (6) Kin: 0.5 mg L^{-1}, NAA: 2 mg L^{-1}; (7) Kin: 0.1 mg L^{-1}, NAA: 5 mg L^{-1}; (8) Kin: 0.2 mg L^{-1}, NAA: 5 mg L^{-1}; (9) Kin: 0.5 mg L^{-1}, NAA: 5 mg L^{-1},(10) BA: 0.1 mg L^{-1}, NAA: 1 mg L^{-1}; (11) BA: 0.2 mg L^{-1}, NAA: 1 mg L^{-1}; (12) BA: 0.5 mg L^{-1}, NAA: 1 mg L^{-1}; (13) BA: 0.1 mg L^{-1}, NAA: 2 mg L^{-1}; (14) BA: 0.2 mg L^{-1}, NAA: 2 mg L^{-1}; (15) BA: 0.5 mg L^{-1}, NAA: 2 mg L^{-1}; (16) BA: 0.1 mg L^{-1}, NAA: 5 mg L^{-1}; (17) BA: 0.2 mg L^{-1}, NAA: 5 mg L^{-1}; (18) BA: 0.5 mg L^{-1}, NAA: 5 mg L^{-1}

(1') Kin: 0.1 mg L^{-1}, IAA: 1 mg L^{-1}; (2') Kin: 0.2 mg L^{-1}, IAA: 1 mg L^{-1}; (3') Kin: 0.5 mg L^{-1}, IAA: 1 mg L^{-1}; (4') Kin: 0.1 mg L^{-1}, IAA: 2 mg L^{-1}; (5') Kin: 0.2 mg L^{-1}, IAA: 2 mg L^{-1}; (6') Kin: 0.5 mg L^{-1}, IAA: 2 mg L^{-1}; (7') Kin: 0.1 mg L^{-1}, IAA: 5 mg L^{-1}; (8') Kin: 0.2 mg L^{-1}, IAA: 5 mg L^{-1}; (9') Kin: 0.5 mg L^{-1}, IAA: 5 mg L^{-1}, (10') BA: 0.1 mg L^{-1}, IAA: 1 mg L^{-1}; (11') BA: 0.2 mg L^{-1}, IAA: 1mg L^{-1}; (12') BA: 0.5 mg L^{-1}, IAA: 1 mg L^{-1}; (13') BA: 0.1 mg L^{-1}, IAA: 2 mg L^{-1}; (14') BA: 0.2 mg L^{-1}, IAA: 2 mg L^{-1}; (15') BA: 0.5 mg L^{-1}, IAA: 2 mg L^{-1}; (16') BA: 0.1 mg L^{-1}, IAA: 5 mg L^{-1}; (17') BA: 0.2 mg L^{-1}, IAA: 5 mg L^{-1}; (18') BA: 0.5 mg L^{-1}, IAA: 5 mg L^{-1}

B5 medium supplemented with 0.1 mg L^{-1} Kin in combination with 5 mg L^{-1} IAA after two months (1.6±0.06 and 1.28±0.07 g respectively). Using leaf explant, the maximum callus fresh weight were obtained in presence of 0.2 mg L^{-1} Kin and 5 mg L^{-1} NAA (1.33±0.06 g). It seems that Kin, as cytokinin, along with 5 mg L^{-1} auxin had most effect on callus growth, which measured as fresh weight. Puricelli *et al*. (2001)

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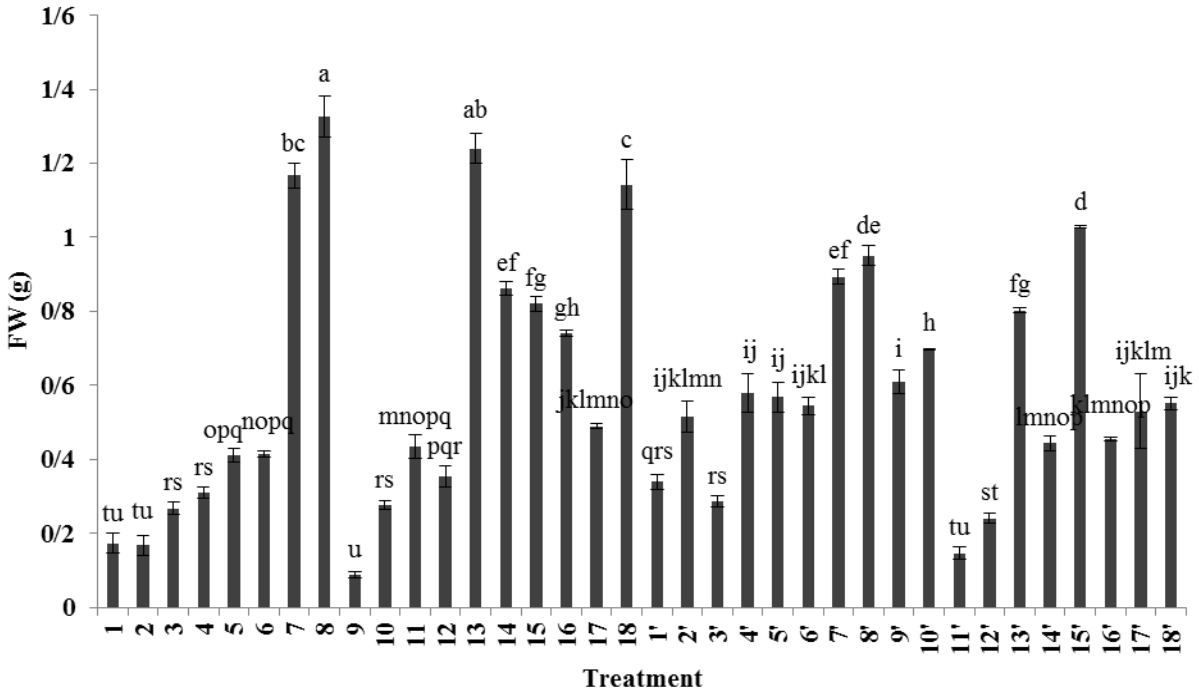

Figure 5. Callus fresh weights (g) under different employed hormonal treatments on leaf explants. The vertical bars represent standard errors. The mean values marked by the same letters are not significantly different at P<0.05 as determined by Duncan's multiple range test.

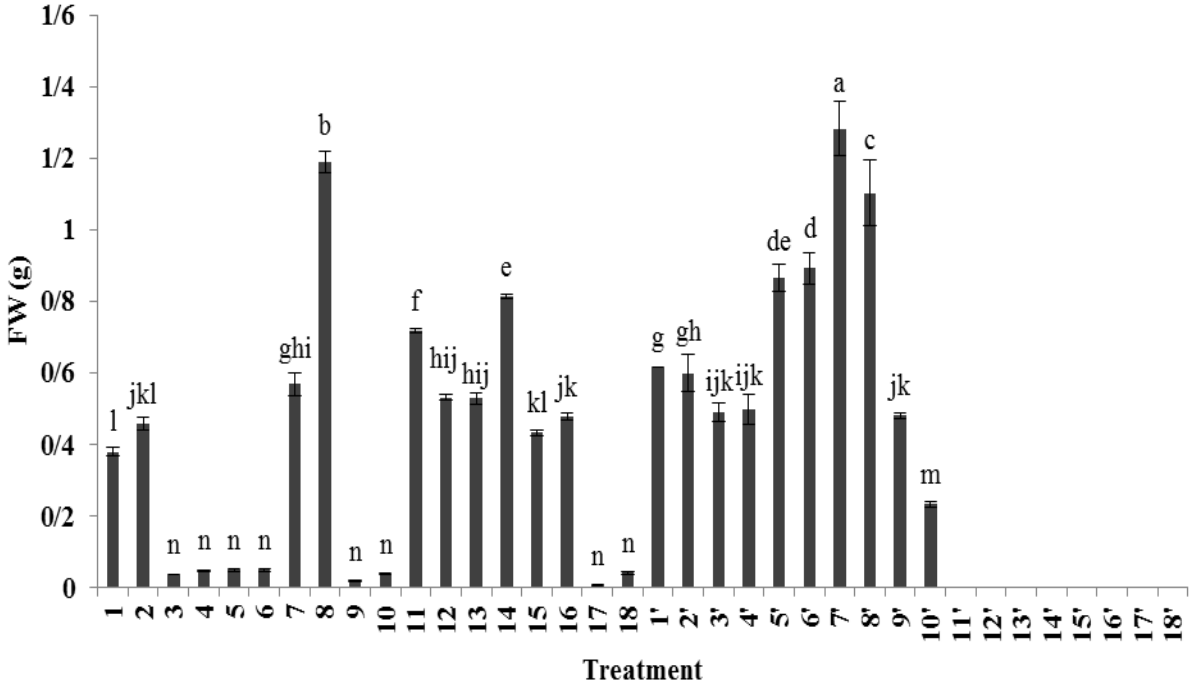

Figure 6. Callus fresh weights (g) under different employed hormonal treatments in root explant. The vertical bars represent standard errors. The mean values marked by the same letters are not significantly different at P<0.05 as determined by Duncan's multiple range test.

used B5 medium supplemented with 2,4-D (2 mg L^{-1}), kinetin (0.2 mg L^{-1}), NAA (0.5 mg L^{-1}) and IAA (0.5 mg L^{-1}) to induce callus in *H. patavinum*. Our results are in good agreement with Abirami and Kumar (2013) and Yaacob *et al*. (2014), which reported the high concentration of auxins and low concentration of cytokinins was more effective for callus formation in *Aegle marmelos* and *Citrus assamensis* respectively. In *C. jambhiri*, among different concentrations of plant growth regulators tested, maximum callus induction observed in presence of 2,4-D alone (Sativa *et al*., 2010; Sativa *et al*., 2011). Similar results have been reported

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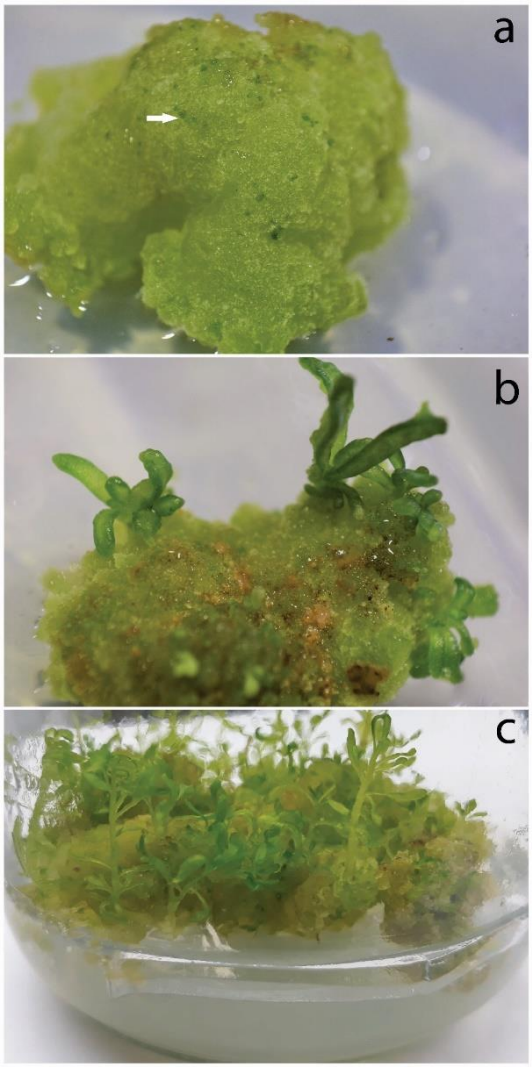

Figure 7. Stages of shoots regeneration from callus culture. (a) Shoot initiation (Green spots on callus that differentiate into shoots), (b) Shoot growth, (c) Shoot multiplication (Increase the number and length of shoots).

in *Ruta graveolens* (Kengar and Paratkar, 2015) and *C. reticulata* (Hasan *et al*., 2016). Also, in *H. tuberculatum* for callus production was used MS medium supplemented with four different concentrations of 2,4-D, 2 and 4 mg L^{-1} 2,4-D induced the highest weight of callus, in comparison to 0.0, 0.5 and 1.0 mg L^{-1} 2,4-D (Elmagrabi *et al*., 2017). However, Kausar *et al*. (2019) reported that the combination of 2.5 mg L^{-1} NAA, 0.25 mg L^{-1} kinetin and 2.5 mg L^{-1} BAP exhibit effective initiation and proliferation of callus in *H. gilesii*. Calli are formed through the modulation of plant hormone signaling, in particular, of auxin and cytokinin. It is known that several key regulators of these hormone signaling pathways (e.g., ARFs and ARRs) function during callus induction (Ikeuchi *et al*., 2013). Here in general, all explants (stem, leaf and root) had the potential to generate callus; However, stem explants found to be more responsive than leaf and root explants. Also, recent studies on rutaceae plants demonstrate similar results, which clearly indicate the callus induction is depending on explant type in *Citrus jambhiri* (Sativa *et al*., 2010) and *C. assamensis* (Yaacob *et al*., 2014). This study also showed that the callus induction in *H. virgatum* as well as the concentration and type of the supplied plant growth regulators depends on the type of cultured explants.

Shoot regeneration: The creamy compact callus which, transferred to shoot induction media, became more greenish and appeared highly competent for shoot bud initiation. First, they appeared as green spots (Figure 7a) then, gradually differentiated to the shoots (Figure 7b) and after two months, numbers and lengths of the shoots were increased (Figure 7c). Various concentrations of BA and Kin (1, 2 and 3 mg L^{-1}) were added in B5 basal medium in order to achieve a fast growing shoots from callus. Lower concentrations of cytokinin (1 mg L^{-1}BA and Kin) in the media did not show any effect on shoot formation. BA at 2 mg L^{-1} concentration produced the maximum number of shoots (38.33±0.88) in stem explants (Table 2). This result was repeated by leaf and root explants, which their shoot number was 22.33±0.33 and 19.33±0.33 respectively (Table 3 and 4). Sativa *et al*. (2010) showed maximum percent of shoot regeneration using nodal segments

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Table 2. The effect of different concentrations of BA and Kin on the number of regenerated shoots per callus induced from stem explant after two months. Values represent means ± SE with three replecated experiments. The mean values marked by the same letters are not significantly different at P<0.05 as determined by Duncan's multiple range test.

Callus Induction Treatment	BA (mg L^{-1})		Kin (mg L^{-1})	
	2	3	2	3
Kin: 0.1 mg L^{-1}, NAA: 1 mg L^{-1}	3.33 ± 0.33^{op}	3.66 ± 0.66^{klmn}	1.33 ± 0.33^{jklm}	2 ± 0^{hij}
Kin: 0.2 mg L^{-1}, NAA: 1 mg L^{-1}	2.33 ± 0.33^{pq}	4 ± 0^{jklm}	0.66 ± 0.33^{klm}	0.33 ± 0.33^{kl}
Kin: 0.5 mg L^{-1}, NAA: 1 mg L^{-1}	4.66 ± 0.33^{no}	5.33 ± 0.66^{ijk}	2 ± 0^{jk}	2.66 ± 0.66^{gh}
Kin: 0.1 mg L^{-1}, NAA: 2 mg L^{-1}	1.66 ± 0.33^{qr}	5 ± 0^{ijkl}	1.33 ± 0.33^{jklm}	1.33 ± 0.33^{hijkl}
Kin: 0.2 mg L^{-1}, NAA: 2 mg L^{-1}	0	1.66 ± 0.33^{nopq}	0.66 ± 0.33^{klm}	1.66 ± 0.66^{hijk}
Kin: 0.5 mg L^{-1}, NAA: 2 mg L^{-1}	2.33 ± 0.33^{pq}	2.66 ± 0.66^{mnop}	1 ± 0^{klm}	0.66 ± 0.33^{jkl}
Kin: 0.1 mg L^{-1}, NAA: 5 mg L^{-1}	4.33 ± 0.33^{no}	4.33 ± 0.66^{jklm}	1.66 ± 0.66^{jkl}	0.66 ± 0.33^{jkl}
Kin: 0.2 mg L^{-1}, NAA: 5 mg L^{-1}	7.66 ± 0.33^{jk}	5.66 ± 0.66^{hijk}	2.66 ± 0.66^{ij}	1.66 ± 0.33^{hijk}
Kin: 0.5 mg L^{-1}, NAA: 5 mg L^{-1}	0	1 ± 0.58^{opq}	0.33 ± 0.33^{lm}	2.33 ± 0.66^{ghi}
BA: 0.1 mg L^{-1}, NAA: 1 mg L^{-1}	0.66 ± 0.33^{rs}	0.33 ± 0.33^{q}	0.33 ± 0.33^{lm}	0
BA: 0.2 mg L^{-1}, NAA: 1 mg L^{-1}	2.33 ± 0.33^{pq}	3.66 ± 0.33^{klmn}	0.66 ± 0.33^{klm}	0.33 ± 0.33^{kl}
BA: 0.5 mg L^{-1}, NAA: 1 mg L^{-1}	5 ± 0^{n}	3 ± 0.58^{lmno}	1.33 ± 0.33^{jklm}	1.66 ± 0.33^{hijk}
BA: 0.1 mg L^{-1}, NAA: 2 mg L^{-1}	3.33 ± 0.33^{op}	6.66 ± 0.66^{ghi}	1.33 ± 0.33^{jklm}	1 ± 0^{ijkl}
BA: 0.2 mg L^{-1}, NAA: 2 mg L^{-1}	5.66 ± 0.33^{lmn}	2.66 ± 0.33^{mnop}	4 ± 0.58^{gh}	3.66 ± 0.66^{fg}
BA: 0.5 mg L^{-1}, NAA: 2 mg L^{-1}	10.33 ± 0.33^{h}	5.66 ± 0.66^{hijk}	4.66 ± 0.66^{fg}	4.33 ± 0.33^{ef}
BA: 0.1 mg L^{-1}, NAA: 5 mg L^{-1}	7.33 ± 0.33^{jk}	5.33 ± 0.88^{ijk}	4.33 ± 0.66^{fgh}	3.66 ± 0.88^{fg}
BA: 0.2 mg L^{-1}, NAA: 5 mg L^{-1}	5.66 ± 0.33^{lmn}	4.33 ± 0.66^{jklm}	0.66 ± 0.33^{klm}	1 ± 0^{ijkl}
BA: 0.5 mg L^{-1}, NAA: 5 mg L^{-1}	0	0.66 ± 0.66^{pq}	1 ± 0^{klm}	0.66 ± 0.33^{jkl}
Kin: 0.1 mg L^{-1}, IAA: 1 mg L^{-1}	16.33 ± 0.33^{e}	5.66 ± 0.66^{hijk}	3.33 ± 0.33^{hi}	2.66 ± 0.33^{gh}
Kin: 0.2 mg L^{-1}, IAA: 1 mg L^{-1}	14.33 ± 0.33^{f}	10.66 ± 0.33^{e}	0	0
Kin: 0.5 mg L^{-1}, IAA: 1 mg L^{-1}'	24.33 ± 0.66^{c}	14.66 ± 0.66^{d}	0	0.33 ± 0.33^{kl}
Kin: 0.1 mg L^{-1}, IAA: 2 mg L^{-1}	15 ± 0.58^{f}	4.33 ± 0.66^{jklm}	0	0.33 ± 0.33^{kl}
Kin: 0.2 mg L^{-1}, IAA: 2 mg L^{-1}	5.33 ± 0.33^{mn}	2.33 ± 0.66^{mnopq}	0	0
Kin: 0.5 mg L^{-1}, IAA: 2 mg L^{-1}	26 ± 1^{b}	19.33 ± 0.66^{bc}	9.66 ± 0.33^{d}	5.66 ± 0.66^{de}
Kin: 0.1 mg L^{-1}, IAA: 5 mg L^{-1}	24.33 ± 0.66^{c}	18.66 ± 0.66^{c}	2.66 ± 0.33^{ij}	1.66 ± 0.33^{hijk}
Kin: 0.2 mg L^{-1}, IAA: 5 mg L^{-1}	38.33 ± 0.88^{a}	25.66 ± 1.2^{a}	1.66 ± 0.33^{jkl}	1.33 ± 0.33^{hijkl}
Kin: 0.5 mg L^{-1}, IAA: 5 mg L^{-1}	9.66 ± 0.33^{hi}	6 ± 0.58^{ghij}	0	0.66 ± 0.66^{jkl}
BA: 0.1 mg L^{-1}, IAA: 1 mg L^{-1}	2.66 ± 0.33^{pq}	8 ± 0.58^{fg}	0	0
BA: 0.2 mg L^{-1}, IAA: 1 mg L^{-1}	6.66 ± 0.66^{klm}	6 ± 1^{ghij}	11.33 ± 0.66^{c}	4.33 ± 0.88^{ef}
BA: 0.5 mg L^{-1}, IAA: 1 mg L^{-1}	13.66 ± 0.66^{f}	10.33 ± 0.33^{e}	5.33 ± 0.33^{f}	4.33 ± 0.88^{ef}
BA: 0.1 mg L^{-1}, IAA: 2 mg L^{-1}	8.66 ± 0.66^{ij}	9 ± 0.58^{ef}	13.66 ± 0.66^{b}	7.66 ± 0.33^{b}
BA: 0.2 mg L^{-1}, IAA: 2 mg L^{-1}	14.33 ± 0.66^{f}	7.66 ± 0.66^{fgh}	9.66 ± 0.33^{d}	6 ± 0.58^{cd}
BA: 0.5 mg L^{-1}, IAA: 2 mg L^{-1}	24.66 ± 0.66^{c}	21 ± 1^{b}	20 ± 0.58^{a}	13 ± 0.58^{a}
BA: 0.1 mg L^{-1}, IAA: 5 mg L^{-1}	18.66 ± 0.66^{d}	9 ± 0.58^{ef}	12 ± 0.58^{c}	7.33 ± 0.33^{bc}
BA: 0.2 mg L^{-1}, IAA: 5 mg L^{-1}	12.33 ± 0.33^{g}	6 ± 1^{ghij}	6.66 ± 0.66^{e}	6 ± 0^{cd}
BA: 0.5 mg L^{-1}, IAA: 5 mg L^{-1}	7 ± 0^{kl}	8 ± 1^{fg}	12.33 ± 0.66^{c}	8.33 ± 0.66^{b}

(71.89%) with NAA (0.5 mg L^{-1}) + BA (1 mg L^{-1}) followed by leaf segments (57%) with NAA (0.5 mg L^{-1}) + BA (3 mg L^{-1}) in *C. jambhiri*. However, callus from root segments did not regenerate shoots. This shows that the type of explant is an important factor for organogenesis in tissue culture (Behera *et al*., 2013). With 2 mg L^{-1} Kin, the highest shoot regeneration in stem, leaf and root explants were 20±0.58, 10.33±0.66 and 1.66±0.33, respectively. Kin also caused the highest regeneration response in the stem explants. 3 mg L^{-1} of both cytokinins reduced the number of shoots compared to 2 mg L^{-1}. However, the highest number of shoots obtained from the stem explants and the lowest number were related to the root explants. Our study showed that the concentration of 2 mg L^{-1}cytokinin was more effective than 3 mg L^{-1}in shoot formation. Multiple reports show that increasing cytokinin concentration is decisive factor for shoot proliferation and elongation, in many medicinal plant species (Rout *et al*., 2000). In *Ruta graveolens*, 2 mg L^{-1} Kin showed the best regeneration response as compared with two other kinetin concentrations (1 mg L^{-1} and 3 mg L^{-1}) (Bohidar *et al*., 2008). Optimum concentrations of Kin studied in *Citrus jambhiri*, was 2 mg L^{-1}which led to a maximum response (Sativa *et al*., 2011). In our study, after evaluating two types of cytokinins, it was revealed the BA was more effective than Kin for initiating shoots and to produce a higher yield of shoot per explants (Table 2, 3 and 4). This is consistent with the results of Ahmad *et al*. (2010) in *Ruta graveolens* and Behera *et al*. (2013) in *Aegle marmelos*. Some studies have shown the use of different concentrations of BA alone to be better treatment for shoot regeneration of different species of Citrus (Raman *et al*., 1992; Costa *et al*., 2002; Sharma *et al*., 2009; Hasan *et al*., 2016) and *Melicope lunu-ankenda* (Rahman *et al*., 2015). A study by

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Table 3. The effect of different concentrations of BA and Kin on the number of regenerated shoots per callus induced from leaf explant after two months. Values represent mean ± standard error of three repeated experiments. The mean values marked by the same letters are not significantly different at P<0.05 as determined by Duncan's multiple range test.

Callus Induction Treatment	BA (mg L^{-1})		Kin (mg L^{-1})	
	2	3	2	3
Kin: 0.1 mg L^{-1}, NAA: 1 mg L^{-1}	1.66 ± 0.33^{nop}	2.66 ± 0.33^{klmn}	0.66 ± 0.33^{hij}	0
Kin: 0.2 mg L^{-1}, NAA: 1 mg L^{-1}	5 ± 0^{ijk}	1.66 ± 0.66^{lmnop}	2.33 ± 0.33^{efg}	2 ± 0.58^{efg}
Kin: 0.5 mg L^{-1}, NAA: 1 mg L^{-1}	2.66 ± 0.33^{mno}	3.66 ± 0.88^{ijkl}	0.66 ± 0.33^{hij}	0.33 ± 0.33^{hi}
Kin: 0.1 mg L^{-1}, NAA: 2 mg L^{-1}	0.66 ± 0.33^{pq}	0.33 ± 0.33^{op}	0.33 ± 0.33^{ij}	0
Kin: 0.2 mg L^{-1}, NAA: 2 mg L^{-1}	0	2.66 ± 0.66^{klmn}	0.66 ± 0.33^{hij}	1.66 ± 0.33^{efgh}
Kin: 0.5 mg L^{-1}, NAA: 2 mg L^{-1}	4.66 ± 0.33^{jk}	4.33 ± 0.33^{hijk}	1.66 ± 0.66^{fghi}	2.33 ± 0.33^{def}
Kin: 0.1 mg L^{-1}, NAA: 5 mg L^{-1}	8.66 ± 0.33^{ef}	6.33 ± 0.66^{fgh}	2.66 ± 0.66^{def}	2 ± 0^{efg}
Kin: 0.2 mg L^{-1}, NAA: 5 mg L^{-1}	9.33 ± 0.33^{e}	5 ± 0^{ghij}	2.66 ± 0.33^{def}	2.33 ± 0.66^{def}
Kin: 0.5 mg L^{-1}, NAA: 5 mg L^{-1}	4.33 ± 0.33	2.66 ± 0.66^{klmn}	0.66 ± 0.33^{hij}	0
BA: 0.1 mg L^{-1}, NAA: 1 mg L^{-1}	0.66 ± 0.33^{pq}	0	0.33 ± 0.33^{ij}	0.66 ± 0.33^{ghi}
BA: 0.2 mg L^{-1}, NAA: 1 mg L^{-1}	2.66 ± 0.33^{mno}	2 ± 1^{lmnop}	1.33 ± 0.66^{fghij}	1 ± 0.58^{fghi}
BA: 0.5 mg L^{-1}, NAA: 1 mg L^{-1}	4.33 ± 0.33^{jkl}	6.33 ± 0.66^{fgh}	1.66 ± 0.66^{fghi}	2 ± 0.58^{efg}
BA: 0.1 mg L^{-1}, NAA: 2 mg L^{-1}	11 ± 0^{d}	10.66 ± 0.66^{bc}	3.66 ± 0.66^{d}	2.66 ± 0.66^{de}
BA: 0.2 mg L^{-1}, NAA: 2 mg L^{-1}	1.66 ± 0.33^{nop}	1.33 ± 0.33^{mnop}	1 ± 0^{ghij}	1 ± 0^{fghi}
BA: 0.5 mg L^{-1}, NAA: 2 mg L^{-1}	7.33 ± 0.33^{fg}	3.66 ± 0.66^{ijkl}	5 ± 0.58^{c}	4.66 ± 0.33^{c}
BA: 0.1 mg L^{-1}, NAA: 5 mg L^{-1}	4 ± 0^{klm}	2.66 ± 0.33^{klmn}	1.66 ± 0.33^{fghi}	2.66 ± 0.66^{de}
BA: 0.2 mg L^{-1}, NAA: 5 mg L^{-1}	6.33 ± 0.33^{ghi}	5.66 ± 0.66^{ghi}	0.33 ± 0.33^{ij}	0
BA: 0.5 mg L^{-1}, NAA: 5 mg L^{-1}	3 ± 0^{lmn}	2.33 ± 0.66^{klmno}	0.66 ± 0.33^{hij}	0.33 ± 0.33^{hi}
Kin: 0.1 mg L^{-1}, IAA: 1 mg L^{-1}	0	2 ± 0^{lmnop}	5.66 ± 0.66^{bc}	5 ± 0^{c}
Kin: 0.2 mg L^{-1}, IAA: 1 mg L^{-1}	22.33 ± 0.33^{a}	11.66 ± 0.88^{b}	0	0
Kin: 0.5 mg L^{-1}, IAA: 1 mg L^{-1}'	5 ± 0^{ijk}	3.66 ± 0.66^{ijkl}	0	1 ± 0^{fghi}
Kin: 0.1 mg L^{-1}, IAA: 2 mg L^{-1}	12.33 ± 0.33^{d}	8 ± 0.58^{def}	0	0
Kin: 0.2 mg L^{-1}, IAA: 2 mg L^{-1}	1.33 ± 0.66^{opq}	3.33 ± 0.66^{jklm}	0	0
Kin: 0.5 mg L^{-1}, IAA: 2 mg L^{-1}	19 ± 0.58^{b}	7 ± 1^{efg}	10.33 ± 0.66^{a}	8 ± 0.58^{a}
Kin: 0.1 mg L^{-1}, IAA: 5 mg L^{-1}	18 ± 1^{b}	9 ± 0^{cd}	0	0.66 ± 0.66^{ghi}
Kin: 0.2 mg L^{-1}, IAA: 5 mg L^{-1}	19.33 ± 0.66^{b}	12.33 ± 0.88^{b}	1.66 ± 0.33^{fghi}	0.66 ± 0.66^{ghi}
Kin: 0.5 mg L^{-1}, IAA: 5 mg L^{-1}	8.33 ± 0.33^{ef}	$5\pm0,58^{ghij}$	0	0
BA: 0.1 mg L^{-1}, IAA: 1 mg L^{-1}	1.33 ± 0.66^{opq}	0.66 ± 0.66^{nop}	0	0.66 ± 0.33^{ghi}
BA: 0.2 mg L^{-1}, IAA: 1 mg L^{-1}	6.66 ± 0.66^{gh}	6.33 ± 0.66^{fgh}	2 ± 0^{efgh}	1.33 ± 0.33^{efghi}
BA: 0.5 mg L^{-1}, IAA: 1 mg L^{-1}	5.66 ± 0.66^{hij}	5 ± 0^{ghij}	3.33 ± 0.33^{de}	2 ± 1^{efg}
BA: 0.1 mg L^{-1}, IAA: 2 mg L^{-1}	16.66 ± 0.88^{c}	15 ± 0^{a}	1.66 ± 0.33^{fghi}	1 ± 0.58^{fghi}
BA: 0.2 mg L^{-1}, IAA: 2 mg L^{-1}	4.33 ± 0.66^{jkl}	6.33 ± 0.66^{fgh}	2 ± 0^{efgh}	2.33 ± 0.33^{def}
BA: 0.5 mg L^{-1}, IAA: 2 mg L^{-1}	11 ± 0.58^{d}	9.66 ± 0.88^{cd}	3.33 ± 0.33^{de}	3.66 ± 0.66^{cd}
BA: 0.1 mg L^{-1}, IAA: 5 mg L^{-1}	6.66 ± 0.33^{gh}	5 ± 1^{ghij}	1.33 ± 0.33^{fghij}	1.66 ± 0.33^{efgh}
BA: 0.2 mg L^{-1}, IAA: 5 mg L^{-1}	5 ± 0^{ijk}	6 ± 1^{fgh}	2.33 ± 0.66^{efg}	2.66 ± 0.88^{de}
BA: 0.5 mg L^{-1}, IAA: 5 mg L^{-1}	8.66 ± 0.66^{ef}	8.66 ± 0.66^{de}	6.66 ± 0.66^{b}	6.66 ± 0.66^{b}

Bohidar *et al*. (2008) on *Ruta graveolens* showed that the media supplemented with BA were most effective in shoot induction, proliferation as well as elongation among all three cytokinins (6-benzyladenine, kinetin, thidiazuron). In another study on the same species, the relative effectiveness of different cytokinins for inducing multiple shoots revealed the order as 6-benzyladenine>2-isopetynyl adenine>kinetin (Faisal *et al*., 2018). The best results were reported by Kausar *et al*. (2019) in shoot proliferation using 3.5 mg L^{-1} BA in *Haplophyllum gilesii*. In addition, the effect of different concentrations of auxin in combination with BA evaluated for shoot regeneration. Ahmad *et al.* (2010) showed the lower concentration of NAA had a synergistic effect with BA and increased the shoot regeneration response while IAA and IBA were less effective in *Ruta graveolens*. In another study on this plant, it was observed that NAA in combination with BA resulted in highest shoot regeneration frequency (Kengar and Paratkar, 2015). However, Sative *et al*. (2011) showed a concentration dependent decrease in shoot regeneration in *C. jambhiri* using 3 mg L^{-1} BA in combination with increasing concentrations of NAA. Our study showed that the shoot regeneration from *H. virgatum* callus culture, not only depends on the explant type, but also affected by the concentration and the type of the previous supplied plant growth regulators for callus induction. The combination of IAA and cytokinin (treatment for callus induction) had profound effect and produced significant induction of shoot system, in comparison with NAA.

Root regeneration: The regenerated shoots were isolated and transferred to ½ MS medium supplemented with NAA, IAA or IBA at different rates. None of the used auxin combinations (Table 5) induced root regeneration in the base of shoots and this area (root

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Table 4. The effect of different concentrations of BA and Kin on the number of regenerated shoots per callus induced from root explant after two months. Values represent mean ± standard error of three repeated experiments. The mean values marked by the same letters are not significantly different at P<0.05 as determined by Duncan's multiple range test.

Callus Induction Treatment	BA (mg L^{-1})		Kin (mg L^{-1})	
	2	3	2	3
Kin: 0.1 mg L^{-1}, NAA: 1 mg L^{-1}	2.33±0.33^{ef}	1.66±0.66^{ef}	0	0.33±0.33^{bc}
Kin: 0.2 mg L^{-1}, NAA: 1 mg L^{-1}	0.33±0.33^{g}	0.33±0.33^{fg}	0	0
Kin: 0.5 mg L^{-1}, NAA: 1 mg L^{-1}	0.66±0.33^{g}	0.33±0.33^{fg}	0.33±0.33^{cd}	0.33±0.33^{bc}
Kin: 0.1 mg L^{-1}, NAA: 2 mg L^{-1}	3.66±0.33^{de}	2.33±0.33^{de}	0.66±0.33^{cd}	0.33±0.33^{bc}
Kin: 0.2 mg L^{-1}, NAA: 2 mg L^{-1}	1.33±0.33^{fg}	1±0^{efg}	0.33±0.33^{cd}	0.66±0.66^{abc}
Kin: 0.5 mg L^{-1}, NAA: 2 mg L^{-1}	1.33±0.33^{fg}	0.66±0.33^{fg}	0.66±0.33^{cd}	0.33±0.33^{bc}
Kin: 0.1 mg L^{-1}, NAA: 5 mg L^{-1}	1±0.33^{fg}	0.66±0.33^{fg}	0.66±0.33^{cd}	1±0^{ab}
Kin: 0.2 mg L^{-1}, NAA: 5 mg L^{-1}	2.33±0.33^{ef}	1±0.58^{efg}	1.33±0.66^{b}	1±0.58^{ab}
Kin: 0.5 mg L^{-1}, NAA: 5 mg L^{-1}	0.66±0.33^{g}	0.66±0.33^{fg}	0.33±0.33^{cd}	1±0.58^{ab}
BA: 0.1 mg L^{-1}, NAA: 1 mg L^{-1}	0	0	0	0
BA: 0.2 mg L^{-1}, NAA: 1 mg L^{-1}	0	0	0	0
BA: 0.5 mg L^{-1}, NAA: 1 mg L^{-1}	0	0	0	0
BA: 0.1 mg L^{-1}, NAA: 2 mg L^{-1}	0	0	0	0
BA: 0.2 mg L^{-1}, NAA: 2 mg L^{-1}	0	0	0	0
BA: 0.5 mg L^{-1}, NAA: 2 mg L^{-1}	0	0	0	0
BA: 0.1 mg L^{-1}, NAA: 5 mg L^{-1}	0	0	0	0
BA: 0.2 mg L^{-1}, NAA: 5 mg L^{-1}	0	0	0	0
BA: 0.5 mg L^{-1}, NAA: 5 mg L^{-1}	0	0	0	0
Kin: 0.1 mg L^{-1}, IAA: 1 mg L^{-1}	13.33±0.33^{c}	6.33±0.66^{c}	1.66±0.33^{a}	1.33±0.33^{a}
Kin: 0.2 mg L^{-1}, IAA: 1 mg L^{-1}	3.33±1.66^{de}	1.66±0.88^{ef}	0	0
Kin: 0.5 mg L^{-1}, IAA: 1 mg L^{-1}'	3.33±1.66^{de}	3.33±0.88^{d}	0	0
Kin: 0.1 mg L^{-1}, IAA: 2 mg L^{-1}	19.33±0.33^{a}	13.33±0.88^{a}	0	1.33±0.33^{a}
Kin: 0.2 mg L^{-1}, IAA: 2 mg L^{-1}	1.33±0.33^{fg}	0.66±0.33^{fg}	0	0.33±0.33^{bc}
Kin: 0.5 mg L^{-1}, IAA: 2 mg L^{-1}	4.66±0.33^{d}	2.33±0.33^{de}	0	0
Kin: 0.1 mg L^{-1}, IAA: 5 mg L^{-1}	4.33±0.33^{d}	3.33±0.88^{d}	1±0.58^{bc}	0.66±0.33^{abc}
Kin: 0.2 mg L^{-1}, IAA: 5 mg L^{-1}	16.33±0.66^{b}	8.33±0.88^{b}	0	0
Kin: 0.5 mg L^{-1}, IAA: 5 mg L^{-1}	2.33±0.33^{ef}	1.33±0.66^{efg}	0	1±0^{ab}
BA: 0.1 mg L^{-1}, IAA: 1 mg L^{-1}	0	0	0	0
BA: 0.2 mg L^{-1}, IAA: 1 mg L^{-1}	0	0	0	0
BA: 0.5 mg L^{-1}, IAA: 1 mg L^{-1}	0	0	0	0
BA: 0.1 mg L^{-1}, IAA: 2 mg L^{-1}	0	0	0	0
BA: 0.2 mg L^{-1}, IAA: 2 mg L^{-1}	0	0	0	0
BA: 0.5 mg L^{-1}, IAA: 2 mg L^{-1}	0	0	0	0
BA: 0.1 mg L^{-1}, IAA: 5 mg L^{-1}	0	0	0	0
BA: 0.2 mg L^{-1}, IAA: 5 mg L^{-1}	0	0	0	0
BA: 0.5 mg L^{-1}, IAA: 5 mg L^{-1}	0	0	0	0

Table 5. Different concentrations of NAA, IAA and IBA used for root induction in the base of regenerated shoots. Each row represents a treatment for root induction

NAA (mg L^{-1})	IAA (mg L^{-1})	IBA (mg L^{-1})
0	0	0
0.5	0	0
1	0	0
2	0	0
0	0.5	0
0	1	0
0	2	0
0	0	0.5
0	0	1
0	0	2
0	0.25	0.25
0	0.25	0.75
0	0.5	0.5
0	0.75	0.25
0	0.5	1.5
0	1	1
0	1.5	0.5

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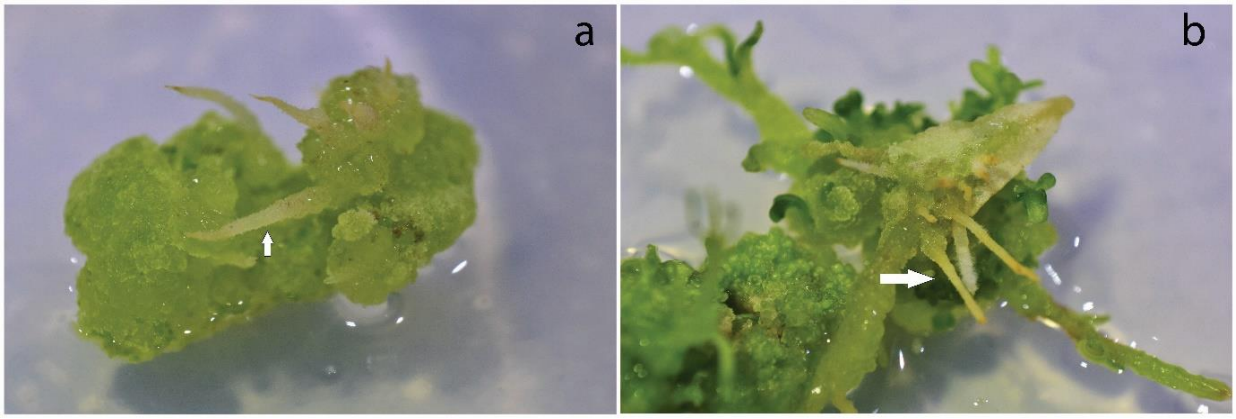

Figure 8. Root regeneration on callus culture from (a) stem explant in B5 medium supplemented with Kin (0.2 mg L^{-1}) and NAA (2 mg L^{-1}), (b) leaf explant in B5 medium supplement with Kin (0.5 mg L^{-1}) and NAA (2 mg L^{-1})

induction from regenerated shoots) requires further studies. However, in some callus-inducing treatments root induction was observed (Figure 8) in presence of Kin (0.1, 0.2 and 0.5 mg L^{-1}) and NAA (2 mg L^{-1}).

Conclusion

In conclusion, the callus and organ regeneration protocols successfully established for *H. virgatum* var. *virgatum*. Stem explants showed the best results in both callus induction and shoot regeneration. Combination of 0.1 mg L^{-1} Kin and 5 mg L^{-1} IAA was the best for callus formation. BA at concentration of 2 mg L^{-1} was the best for shoot induction and regeneration using callus culture. The protocol developed may aid the sustainable production of the phytochemical and planting material. Due to *H. virgatum* var. *virgatum* is an endemic rare species and grows in a narrow geographical area, a big step has been taken in the micropropagation of this species and this research needs to be continued.

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