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AN EFFICIENT AND IMPROVED MICROPROPAGATION METHOD OF RUMEX NERVOSUS: A VALUABLE MEDICINAL PLANT FROM SAUDI ARABIA

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Abstract

Rumex nervosus, a large annual herb is a member of the Polygonaceae family, It is an edible medicinal plant that shows antimicrobial and antioxidant activities and used in traditional medicine to treat various ailments. In this study, a new and improved *in vitro* propagation protocol was developed for *R. nervosus.* After 1 week of inoculation on plant growth regulator-free Gamborg's B-5 basal (B5) medium, significant seed germination reached 90%. The highest shoot multiplication rate was observed on B5 medium supplemented with 0.5-µM benzylaminopurine (BAP); 7.8 shoots per explant). The maximum mean number of roots was obtained on B5 medium with 0.5-µM 1-naphthaleneacetic acid (maximum number of roots: 29; mean value of root length: 5 cm). Plants were successfully acclimatized into plastic pots containing sterile soil and sand at a 1:1 ratio. Callus induction was best supported on Murashige and Skoog (MS) medium supplemented with 2,4-dichlorophenoxyacetic acid (2.5 µM) and BAP (0.1 µM).

Key words: *Rumex nervosus*, Micropropagation, *In vitro*, Tissue culture, Adaptation.

Abbreviations: NAA: 1-Naphthaleneacetic acid; **2,4-D**: 2,4-Dichlorophenoxyacetic acid; **BAP**: Benzylaminopurine; **MS**: Murashige and Skoog medium; **WPM**: Woody plant medium, Lloyd and McCown; **B5**: Gamborg's B-5 basal medium, developed by Gamborg, *et. al*.; **IAA:** Indole acetic acid; **IBA:** Indole-3-butyric acid**; PGRs:** Plant growth regulators.

Introduction

The genus *Rumex*, with more than 200 species of the Polygonaceae family, is found at an altitudes above 1,000 m asl (Nigussie, 2020; Alasmari, 2020). *Rumex nervosus* is a large annual herb it bears a nut that is light brown and fruits that are cordateorbicular (Al Yahya *et al*., 2018; Aati *et al*., 2019). The plant is commonly found in the eastern part of Africa and in the Arabian Peninsula. *Rumex nervosus* is an edible plant; however, because of its high calcium oxalate content, it can produce toxic effects if consumed in large amounts (Jaya & Yesuf, 2010; Alzoreky & Nakahara, 2003). Furthermore, it is a medicinal plant used in traditional medicine to treat stomach disorder and pain, wounds, ophthalmic conditions, dysentery, diarrhea, and pharyngitis (Khan *et al*., 2018; Sher & Alyemeni, 2011; Ghebremariam *et al*., 2018). The leaves of the plant are boiled in water and the filtered water extract is consumed for the treatment of diarrhea; similarly, it is used against wounds, eczema, typhus, and rabies (Asad *et al*., 2004). *Rumex nervosus* shows various biological activities (Desta *et al*., 2015; Quradha *et al*., 2019). A portion extracted with chloroform has activity against MCF-7 and MDA-MB-231 cell lines (Alasmari, 2020). Several important compounds such as flavonoids, anthraquinones and gallic acid are reported. The methanolic extracts of the roots and leaves of the plant are effective against helminthiasis and diarrhea, as well as shows notable antimicrobial activity (Al-Asmari *et al*., 2015; Tedila & Assefa, 2019). In a previous study, methanolic extract from *R. nervosus* leaves showed both antioxidant and antimicrobial activities (Al-Asmari *et al*., 2015), *Rumex* leaves have also shown to contain antioxidant and anticancer compounds (Alasmari, 2020). In general, natural antioxidants found in plants are being increasingly utilized for their nutritional value and safety. Moreover, reports indicate that >80% of the world's population depends on traditional medicines for their primary healthcare needs (Al-Nowihi *et al*., 2020). Therefore, improving propagation methods for medicinal plants is more important than ever. Van Assche *et al*., (2002) demonstrated in a seed germination study of different *Rumex* species that only 2 species out of 9 germinated after 6 months storage indicating that seeds were short lived or germination required specific environment. In another study, *Rumex* seeds were found to be highly dormant (46%–99%). These potential issues can be overcome by micropropagation in the laboratory (Al Khateeb *et al*., 2017). Indeed, the production of biologically active entities can be optimized for the enrichment of plants and the production of genotypic elite groups (Chavan *et al*., 2014). Over recent decades, *In vitro* reproduction has contributed to the growth of the pharmaceutical industry through studies of the biosynthetic capabilities of plant cells to obtain beneficial products, cultivar enhancement, production of secondary metabolites, and plant metabolism (Altpeter *et al*., 2016; Cruz-Cruz *et al*., 2013). *Rumex nepalensis* is an important source of secondary metabolites in addition to being used in folk remedies; therefore, it is considered an important medicinal herb. The efficacy of *In vitro* regeneration of this plant was tested experimentally by Bhattacharyya *et al*., (2017) via direct organic formulation and indirect organic formation. The regeneration methodology, plant fraction, and solvent system greatly influence levels of various secondary metabolites such as phenols, flavonoids, alkaloids, and tannins. An improved propagation method was reported by Manoj *et al*., (2019). for *Rumex vesicarius*, which required an efficient *In vitro* production system because of its

medicinal importance and limited reproductive capacity. In addition, Ślesak *et al*., (2014) attempted to develop an effective and fast method for regenerating *Rumex tianschanicus* × *Rumex patientia* because the hybrid showed high levels of bioactive substances. To date, however, a propagation protocol has yet to be developed for *In vitro* mass propagation of *R. nervosus*. Therefore, the development and optimization of such an *In vitro* method were investigated in this study.

Materials and Methods

Plant material: The seeds of *R. nervosus* were collected during autumn 2020, specifically from the Wadi Ghazal in the Taif region of the Kingdom of Saudi Arabia (Fig. 1).

Fig. 1. *Rumex nervos*us.

Fig. 2. *Rumex nervosus* seeds after 1 week of growth having been treated with 70% Clorox for 15 min.

Media for seed germination: *In vitro* cultures of *R. nervosus* were established from seeds. Seeds were washed with tap water and a few drops of Tween-20. Then the seeds were sterilized with 50% or 70% Clorox for 15 or 30 min (Table 1). Finally, seeds were rinsed with sterile distilled water three times (3 min per wash) to remove excess of bleach. Sterilized seeds were inoculated on media Murashige and Skoog (MS) (Murashige and Skoog., 1962), woody plant medium (WPM) (McCown., 1981) , and Gamborg's B-5 basal (B5) medium (Gamborg *et al*., 1968) as well as in without plant growth regulators (PGRs) as control. The pH of the media was adjusted to 5.8. In addition, 7.0 g/L of agar was added to solidify the media and 25g of sucrose and essential vitamins were added. Media were then autoclaved at 121°C for 20 min. Subsequently, cultures were incubated in a growth chamber at 25 ± 2°C with 80% humidity. Light conditions were 16 hours light and 8 hours darkness with cool fluorescent tube light at 40 µmol m^2s^{-1}.

Shoot proliferation: The shoot and nodal parts (approximately 2cm) of *In vitro* grown seedlings were used as starting materials. A mix of nodal segments (Shoot tips/nodal segments) were excised and cultured on B5 medium supplemented with benzylaminopurine (BAP) at 0.5, 1.0, and 2.0 µM. Data were collected after 4 weeks of culture for the number of leaves, number of shoots, and root formation.

Root regeneration: Shoot portions (approximately 2 cm) were used as explants for the rooting experiment. Four rooting PGRs, 2,4-dichlorophenoxyacetic acid (2,4-D), 1-naphthaleneacetic acid (NAA), indole-3-butyric acid (IBA), and indole acetic acid (IAA) were supplemented to MS medium at concentrations of 0.5, 1.0, and 2.0 µM to test their effects on root induction. The *In vitro* rooting percentage, average number of roots, and average root length (cm) were measured after 4 weeks of culture.

Callus induction: Leaf parts (approximately 1 cm^2) and stem segments from *In vitro* grown seedlings of *R. nervosus* were used as starting materials for callus induction. Different levels of BAP, 2,4-D, NAA, and Kin were added to growth media in various combinations. The media were further fortified with 100mg of adenine sulfate, 100mg of glutamic acid, and 1g of polyvinylpyrrolidone (PVP). The data on callus induction rate were collected after 4 weeks of culture.

Acclimatization: Plants were carefully removed from boxes, regenerated, thoroughly washed, and placed to pots with soil and sand mixed in a 1:1 ratio to acclimate them.Plants were first acclimated for two weeks at 25 2°C and then exposed to light for 16 hours.Before being relocated to a glass house (temperature: 25°C; humidity: 70%; light intensity: 50%), the plants were watered with half MS salt solution without sucrose.

Results

Seed germination and shoot proliferation: Seeds were inoculated on MS, B5, and WPM media free of PGRs. The best sterilization effects were obtained using 70% Clorox for 15min (Table 1); 90% of inoculated seeds began to germinate after 1 week (Fig. 2). Growth in B5 medium resulted in the most branches (five to six buds each) (Fig. 3). To identify the most effective level of cytokinin for plant reproduction, BAP at 0.5, 1.0, and 2.0µM was tested. Seedlings grown on medium supplemented with 0.5µM BAP showed the maximum no of shoots (7.33 shoots /explant) and longest shoots average (3.66 cm) as well as the optimum no of leaves (13.66 leaves /plant) (Table 2 and Fig. 4); however, increasing levels of BAP to more than 0.5µM reduced the number of shoots and number of leaves with other higher concentrations of BAP The minimum number of shoots/length/no of leaves was recorded from control (without any plant growth regulator). Therefore a PGR was necessary to induce multiplication.

Table 1. The impact of different disinfection conditions on the germination rate of *Rumex nervosus.*

Number	Disinfection time (min)	Clorox (%)	Number of seeds	Pollution rate (%)	Germination rate (%)
1	15	50	10	20	50
2	15	70	10	0	90
3	30	50	10	10	40
4	30	70	10	0	90

Table 2. Effects of the plant growth regulator (PGR) BAP on the shoot proliferation and leaf number of *Rumex nervosus* after 4 weeks of growth.

Concentration of PGR (µM)	Average number of shoots ± SE	Average length of shoots	Average of number of leaves ± SE
BAP (0.0)	2.66 ± 0.57^{c}	3.00 ± 0.50^{a}	6.3 ± 1.52^{c}
BAP (0.5)	7.33 ± 0.57^{a}	3.66 ± 0.76^{a}	$1.52^{a} \pm 13.66$
BAP (1.0)	6.33 ± 0.57^{ab}	3.33 ± 0.57^{a}	$0.00^{b} \pm 10.00$
BAP (2.0)	4.66 ± 1.52^{b}	2.50 ± 0.50^{a}	8.66 ± 1.15^{b}

The experimental design included four replications per treatment and four explants per replication. After 4 weeks of culturing, mean ± standard error (SE) values were calculated. ^{a,b,c} Means within the same column with different superscripts differ significantly ($p<0.05$)

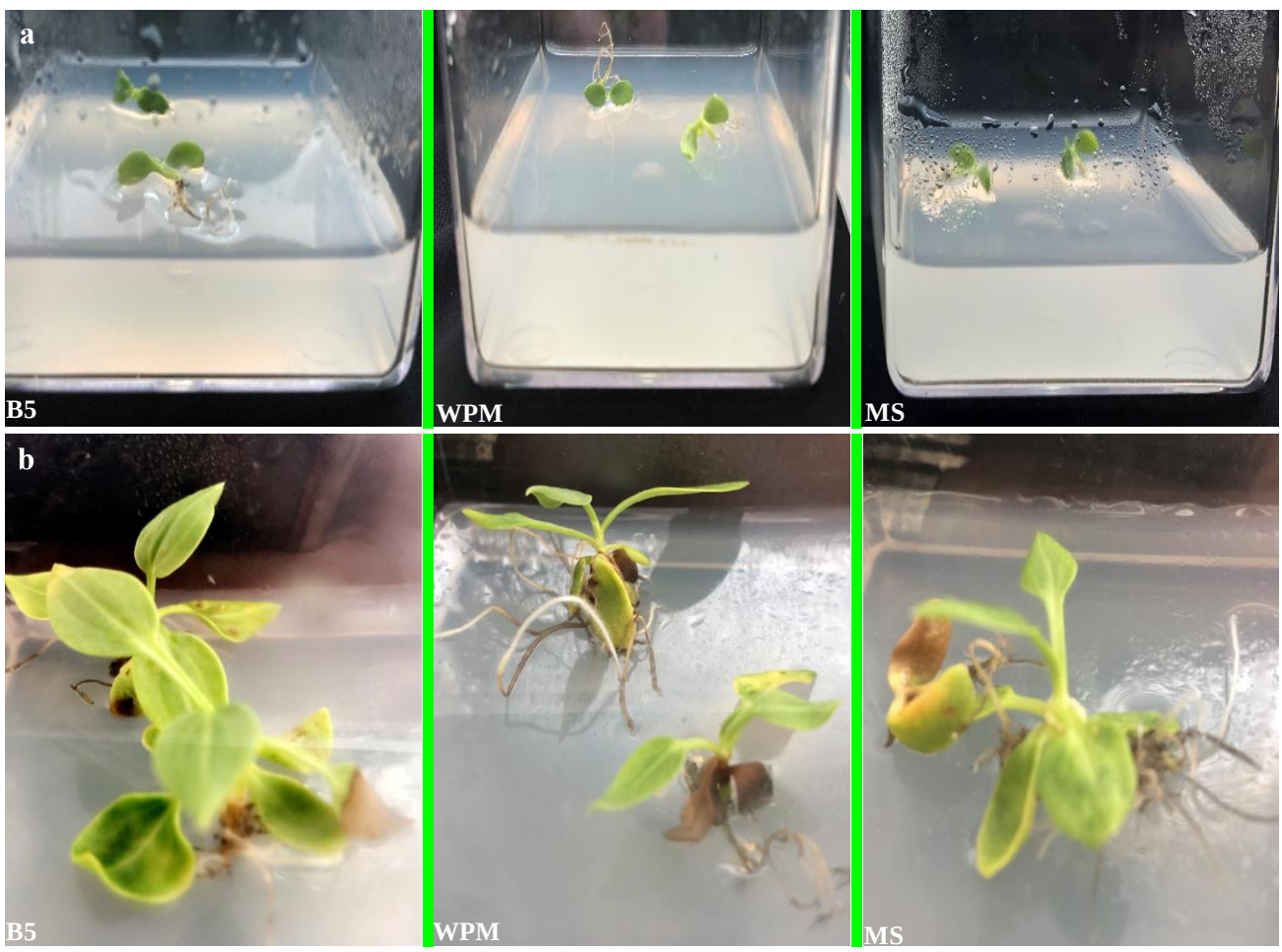


Fig. 3. Effect of different media on plant growth in *Rumex nervosus*. (a) The first day in the media. (b) After 1 month in the media.

Root number and length: Different levels of IAA, IBA, and NAA were applied to test the effects of PGR types on root formation. The multiplied shoots were cut individually and inoculated on B5 medium with different concentration (0.5, 2.5, and 5.0 µM) of the four PGRs (Figs. 5–7). The number and length of roots were recorded after 4 weeks of culture. Shoots grown in B5 medium with 0.5-µM NAA showed the highest number of roots (30 roots per plant) with the longest length of (5.75) cm (Figs. 8-9). Plants with good roots were successfully transferred to plastic pots containing sterile soil and sand in a 1:1 ratio. The survival rate of these plants were 100 % at 60 days after being transferred to the pots (Fig. 10).

Callus induction: Leaf and stem segments were also grown on MS media supplemented with various levels of 2,4-D (0.0, 2.5, 3.0, 4.0, and 5.0 µM), BAP (0.1 and 0.5 µM), NAA (0.1 and 0.5 µM), and Kin (0.5 and 1.0

µM) and control. The control media did not induce callus formation on media without any PGRs, while the best callus induction was obtained with 2.5-µM 2,4-D in combination with 0.1-µM BAP which was supplementd with 100-mg adenine sulfate, 100-mg glutamic acid, and 1-g PVP in MS media. Thus the calluses obtained after 4 weeks of culture were soft and yellow colour. The highest fresh weight was observed 5.86g, on MS media 2.5-µM 2,4-D in combination with 0.1-µM BAP the color of calluses were yellowish white, induced from leaf explants, and the texture of these calluses was friable (Table 3 and Fig. 11).

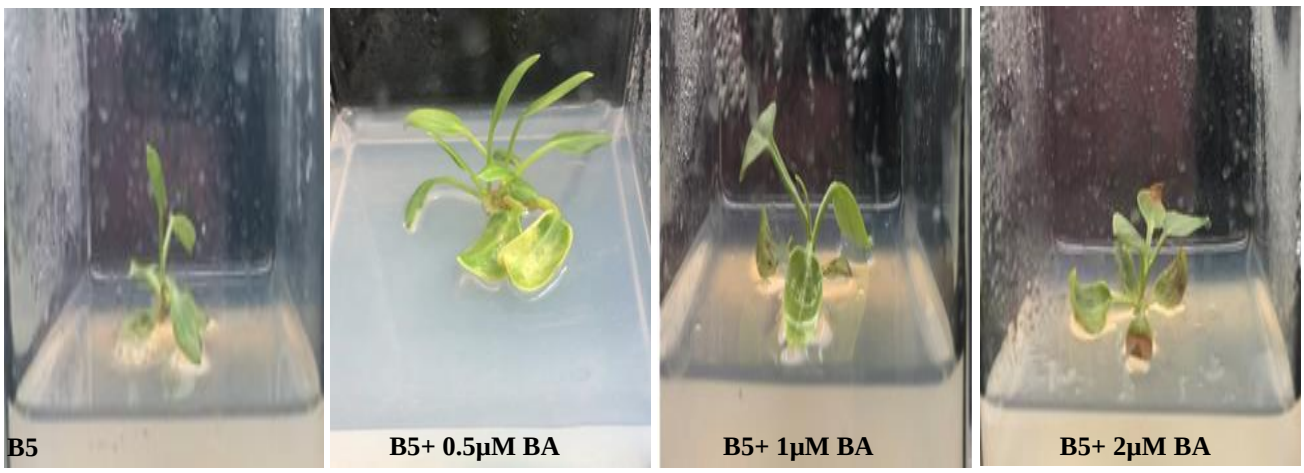


Fig. 4. Effects of different concentrations of BA supplemented to B5 media on plant growth in *Rumex nervosus*.

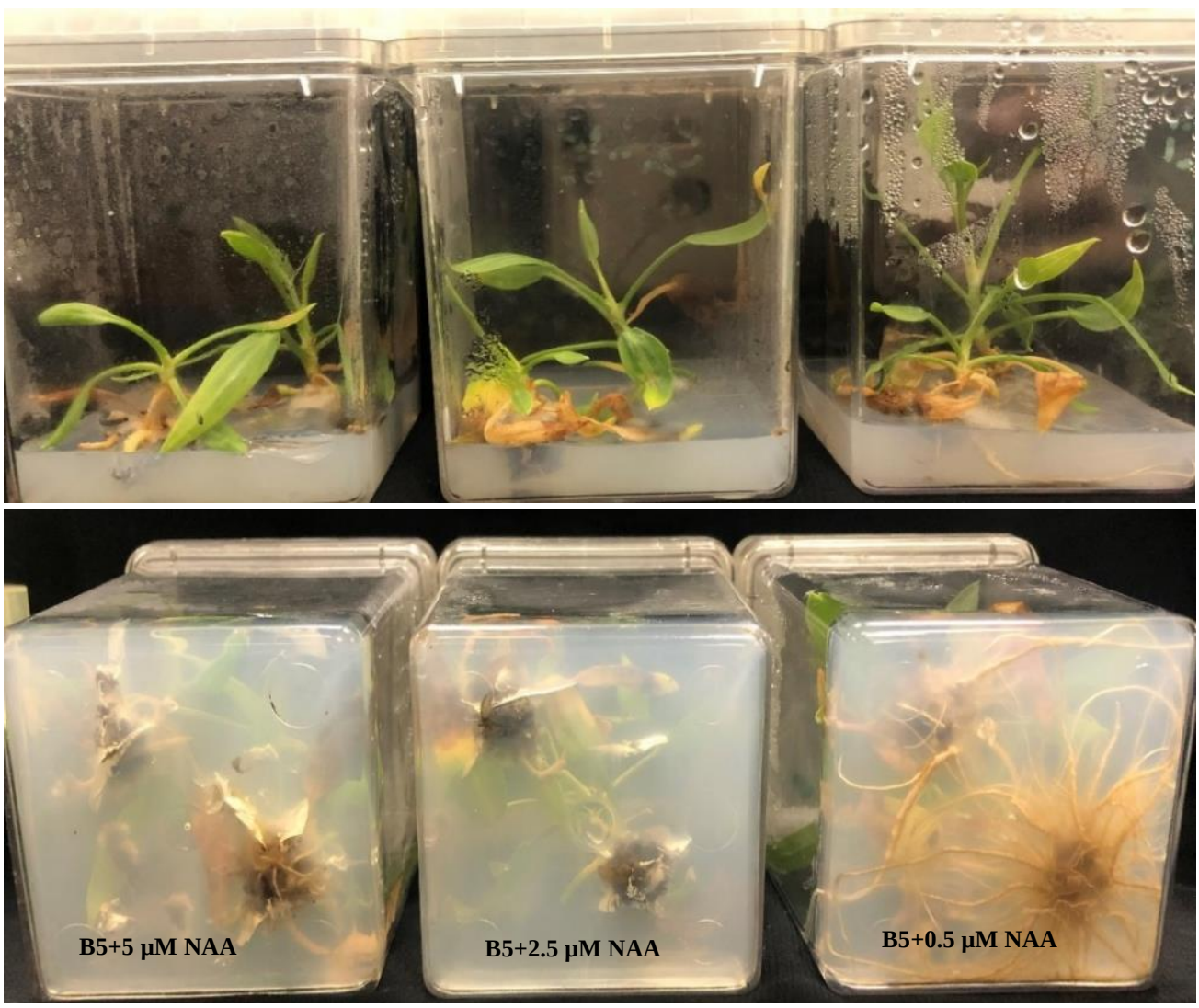


Fig. 5. Effects of different concentrations of NAA supplemented to B5 media on root formation in *Rumex nervosus*.

Table 3. Effect of different plant growth regulator (PGR) combinations on the callus formation of *Rumex nervosus* at 1 month after inoculation.

Treatment	Plant growth regulator (µM)				Callus formation	Leaf Callus (g)	Stem callus (g)
	2,4-D	BAP	NAA	Kin			
MS1	0.5	0.5	0.0	0.0	−	0.0	00
MS2	0.5	0.0	0.0	0.1	−	0.0	00
MS3	1.0	0.1	0.0	0.0	−	0.0	00
MS4	2.0	0.1	0.0	0.0	−	0.0	00
MS5	2.0	0.0	0.1	0.0	−	0.0	00
MS6	2.5	0.1	0.0	0.0	+	5.8	2.0
MS7	2.5	0.5	0.0	0.0	+	3.5	1.5
MS8	2.5	0.0	0.5	0.0	−	0.0	00
MS9	2.5	0.0	0.0	1.0	−	0.0	00
MS10	5.0	0.5	0.0	0.0	−	0.0	00
MS11	5.0	0.0	0.0	1.0	−	0.0	00
MS12	10.0	0.1	0.0	0.0	−	0.0	00
MS13	10.0	0.5	0.0	0.0	−	0.0	00
MS14	10.0	1.0	0.0	0.0	−	0.0	00

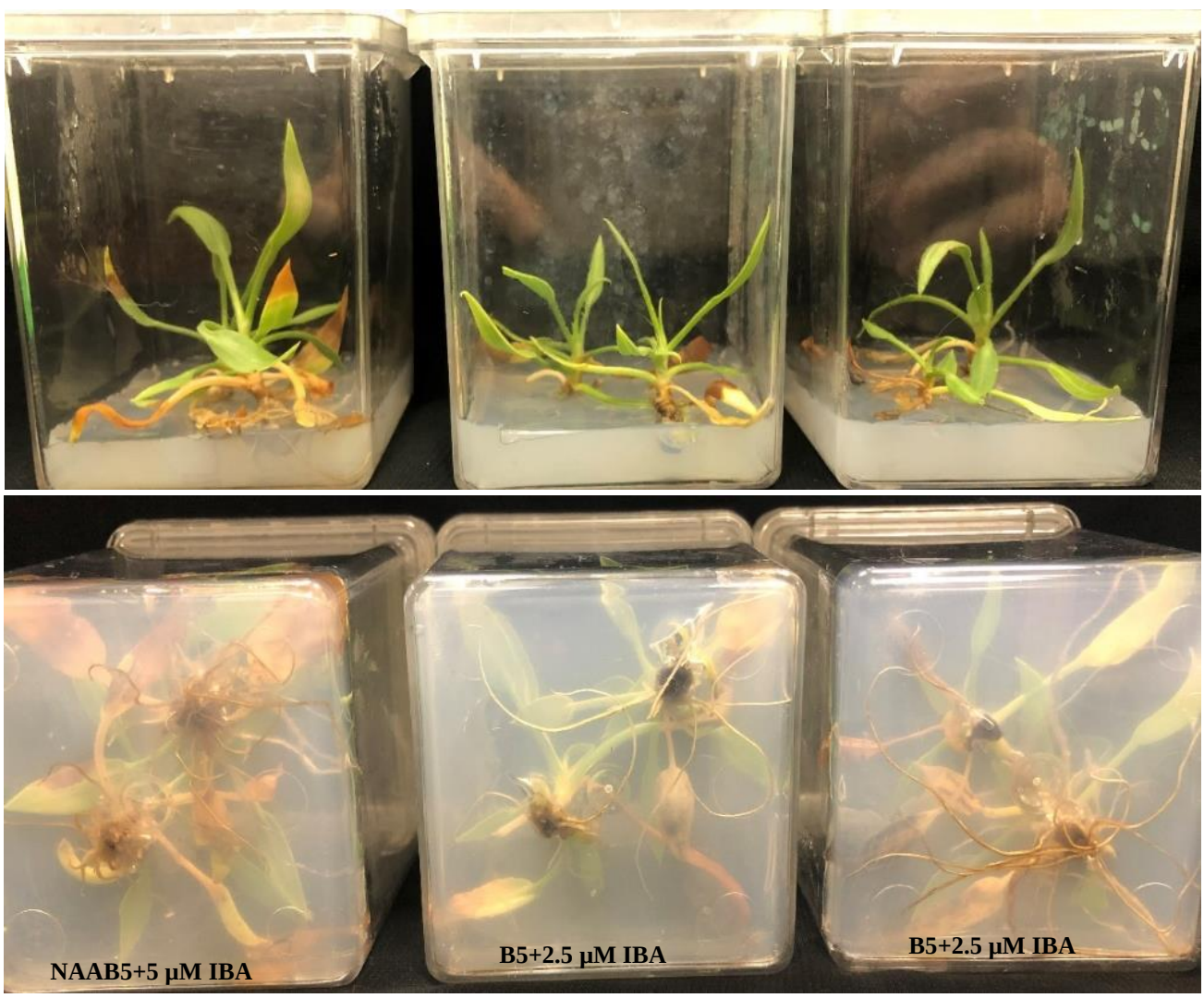


Fig. 6. Effects of different concentrations of IBA supplemented to B5 media on root formation in *Rumex nervosus*.

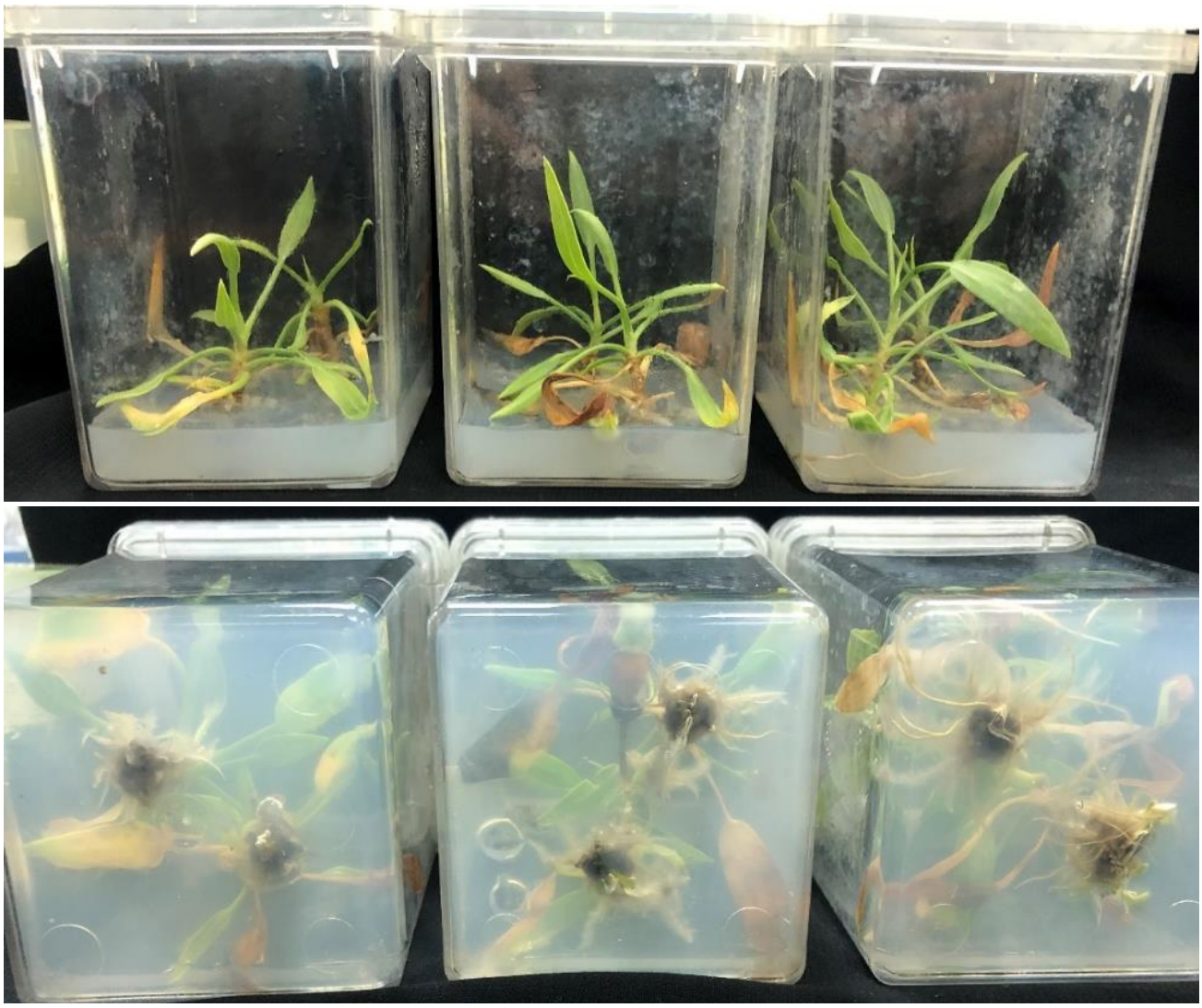


Fig. 7. Effects of different concentrations of IAA supplemented to B5 media on root formation in *Rumex nervosus*.

Discussion

The aim of the current study was to identify a standardized protocol for *Rumex nervosus* propagation in the laboratory. In fact it was the first study to develop an effective micropropagation protocol for this important medicinal plant species, having potential antioxidant, cytotoxic, antifertility, antimicrobial, antiinflammatory, antidiarrheal, and antiviral properties (Nigussie, 2020; Qaid *et al*., 2021). Overall, *R. nervosus* seed germination was 50% successful. Higher seed germination rates were observed in B5 medium than those observed in MS medium and WP medium, possibly due to the lower percentage of NO_3 and NH_4 in B5 and the higher percentage of potassium and magnesium, affecting the nitrogen (Russowski *et al*., 2006). In *R. nervosus* grown in soils with high concentrations of potassium, calcium, and magnesium and a low concentration of nitrogen, the leaves contained a large amount of potassium, calcium, and magnesium (Alghamdy, 2013). Growth regulators, especially cytokinins, are considered an important factor for shooting proliferation (Al Khateeb *et al*., 2017). In the present study, bud growth on B5 medium supplemented with BAP resulted in branches being well spread. The most common problem *In vitro* was callus browning, which might be due to the oxidation of polyphenols; therefore, 1 g/L of PVP and ascorbic acid were added separately to MS medium containing 2.5 µM of 2-4,D and 0.1 µM of BAP. PVP reduced the appearance of browning; its inclusion resulted in yellow friable calluses similar to those of *R. vicarious* (Manoj *et al*., 2019). This result was also consistent with that of El-Shafey *et al*., (2019), who reported that *Rumex pictus* favored a higher concentration of 2,4-D in culture medium compared with that of BAP to increase flavonoid levels in calluses. The addition of ascorbic acid delayed the formation of calli and decreased the formative response, which was similar to the results of Ślesak *et al*., (2014). Furthermore, the addition of glutamic acid also supported the stimulation of callus induction (Sun & Hong, 2010). Adenine sulfate can also play a role in the mass reproduction of plants (Singh *et al*., 2017). The stimulation of roots from shoots multiplied *In vitro* is essential for the success of a micropropagation protocol. Previously, the most effective auxins for rooting were found to be IBA and NAA (Al Khateeb *et al*., 2017). In the current study, a low concentration of NAA produced a high rooting rate and healthy roots. After sufficient development of roots, plants were successfully transplanted into plastic pots with sterile soil and sand at 1:1 ratio. The survival rate of the transplanted plants was 100%.

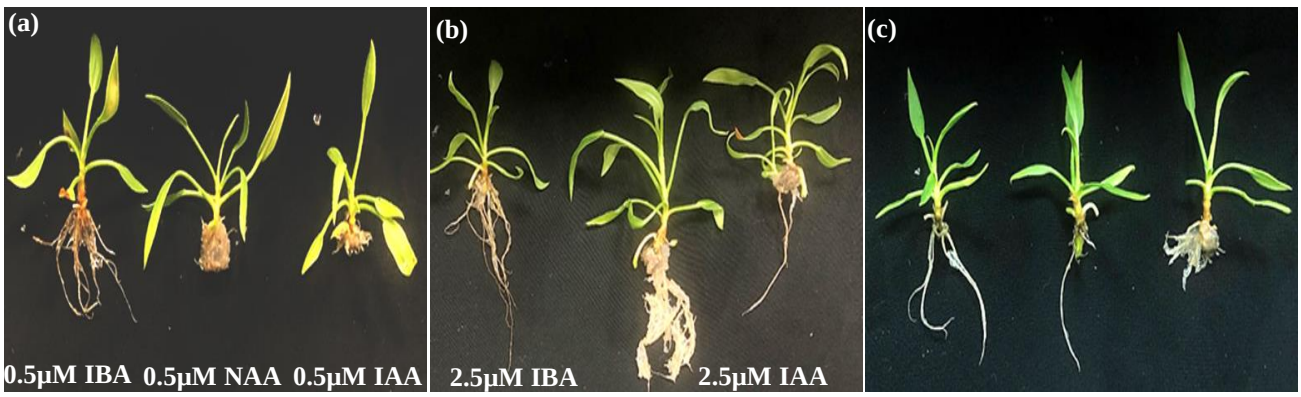
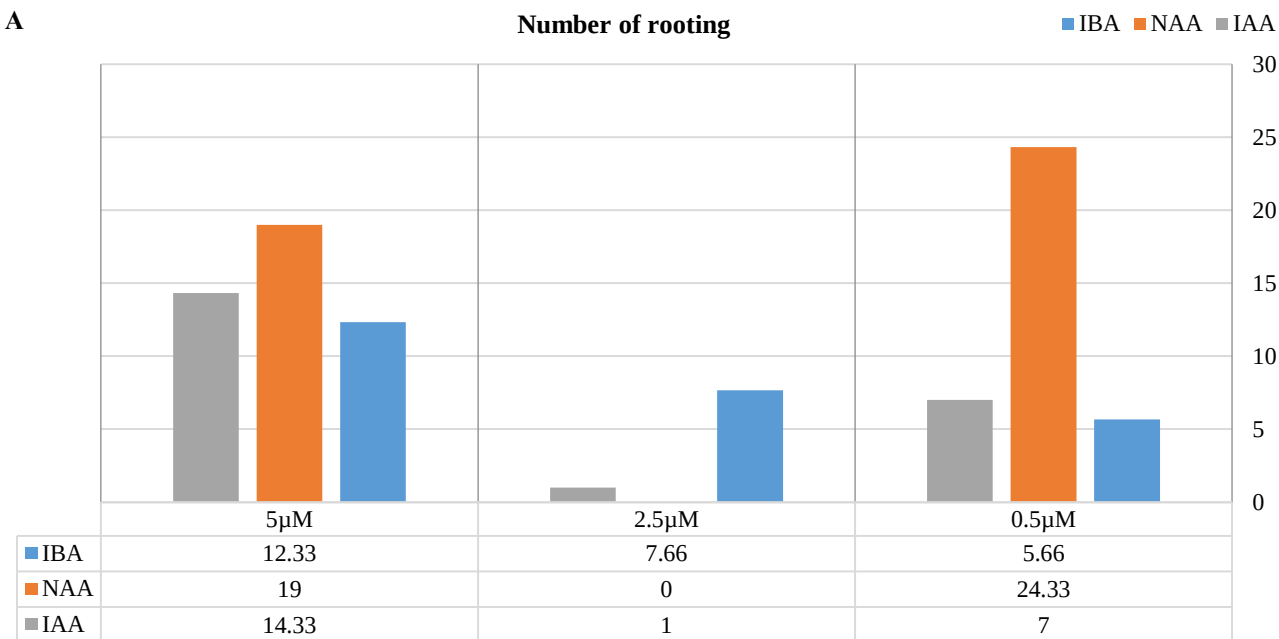
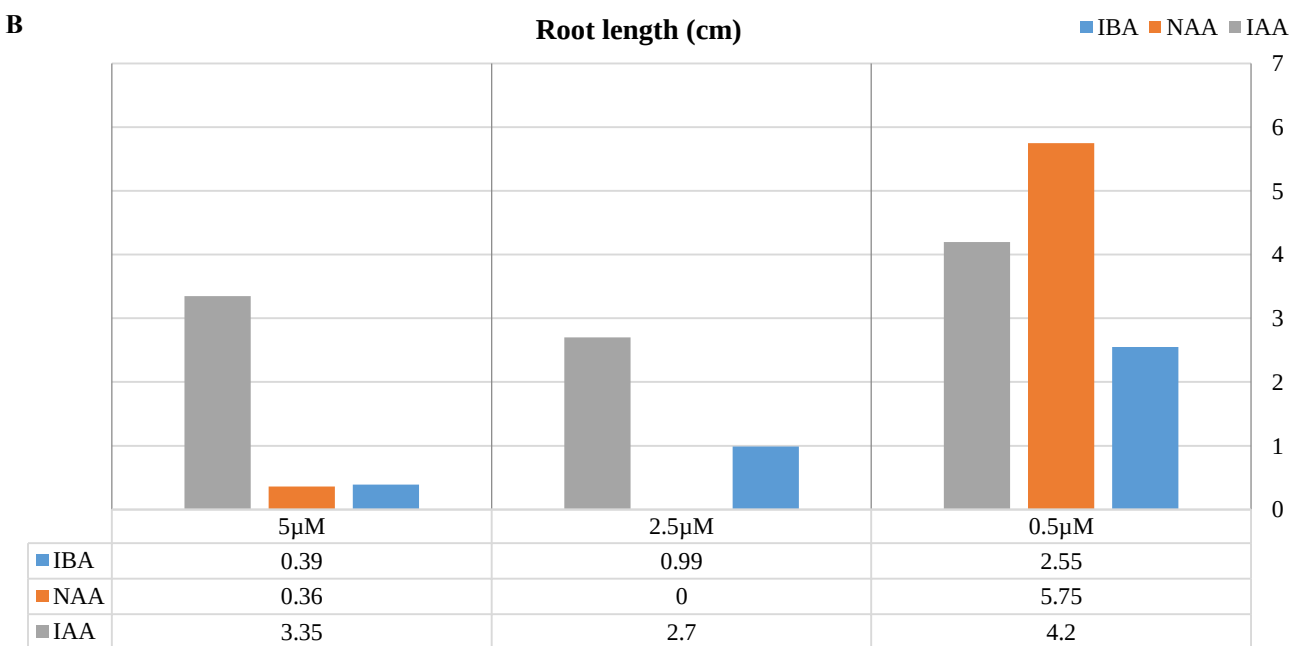


Fig. 8. Effects of (a) 0.5 mg/L, (b) 2.5 mg/L, and (c) of 5 mg/L of IBA, NAA, and IAA on root formation in *Rumex nervosus*.



	5µM	2.5µM	0.5µM
IBA	12.33	7.66	5.66
NAA	19	0	24.33
IAA	14.33	1	7



	5µM	2.5µM	0.5µM
IBA	0.39	0.99	2.55
NAA	0.36	0	5.75
IAA	3.35	2.7	4.2

Fig. 9. Proliferation of *Rumex nervosus* rooting. (A) Effects of IBA, NAA, and IAA on a number of roots. (B) Effects of IBA, NAA, and IAA on root length. Data represent mean values ± standard error of 10 replicates.

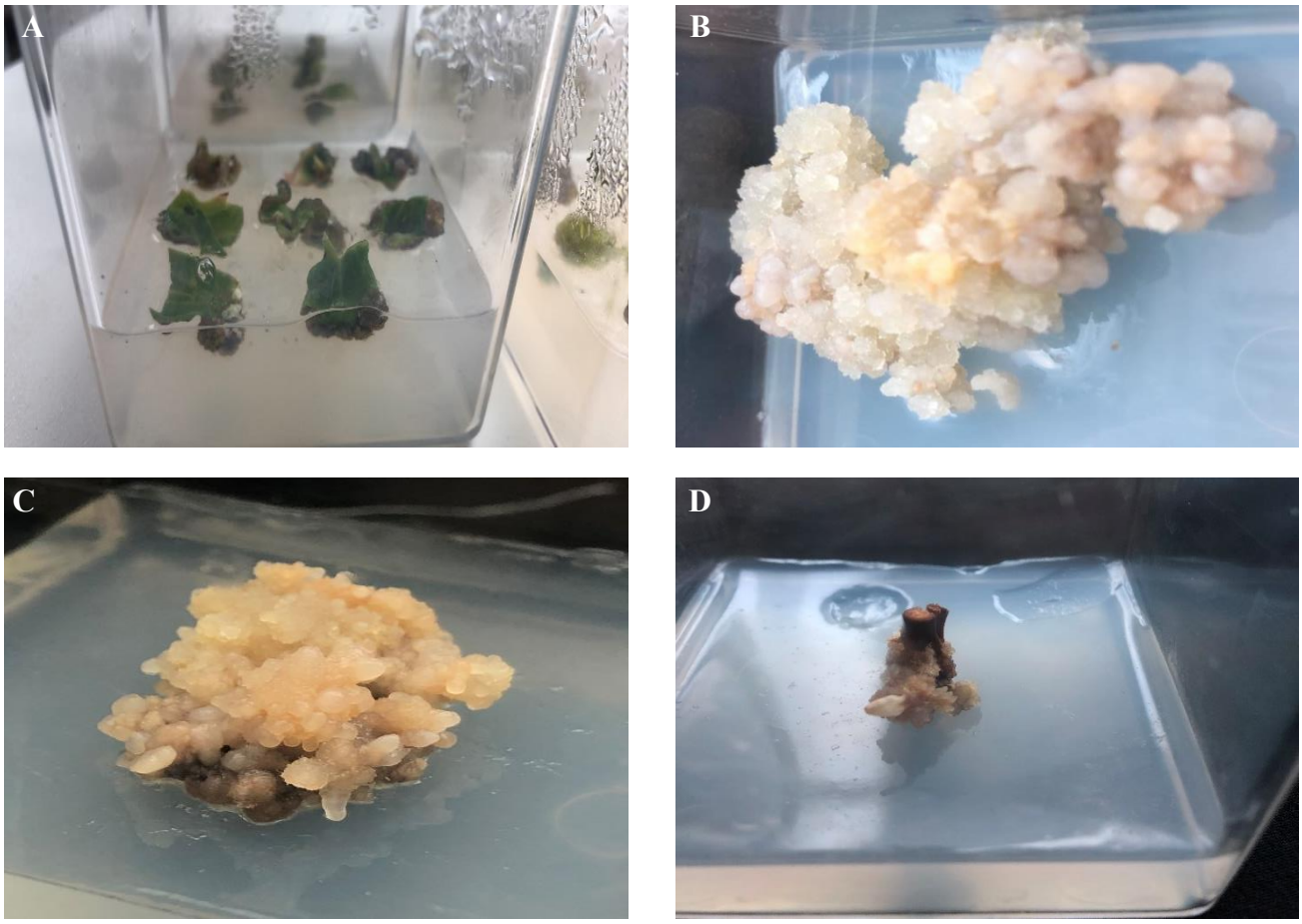


Fig. 11. *Rumex nervosus* callus grown on MS medium supplemented with 2.5-µM 2,4-D and 0.1-µM BAP induced from leaf (a,b) and nodal (c,d) explants.

Fig. 10. Acclimatization of *Rumex nervosus* after 3 weeks of growth in soil with a 1:1 mixture of sterile soil and sand.

Conclusions

Previously, the *In vitro* multiplication of *R. nervosus* from microcuttings had not been reported, nor had callus induction been documented in this species. The results of this study therefore provide new data on the micropropagation of *R. nervosus*. Indeed, we provide an effective protocol for *In vitro* propagation of this important medicinal plant. These micropropagation and callus induction techniques will help to provide the raw materials for secondary metabolite production and to conserve the plant.

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