In vitro regeneration of pyrethrum (Chrysanthemum cinerariaefolium) plantlets from nodal explants of in vitro raised plantlets

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ABSTRACT

Limited access to high quality planting material is a major factor constraining productivity of pyrethrum in Rwanda. The objective of this study was to develop an efficient protocol for regeneration and mass propagation of high yielding and high pyrethrin content pyrethrum cultivars using tissue culture techniques. Nodal explants from in vitro grown pyrethrum plantlets were cultured onto Murashige and Skoog (MS) media supplemented with different concentrations of cytokinins 2-isopentyl adenine (2iP), Benzylaminopurine (BAP), Kinetin (KIN), Thidiazuron (TDZ), cysteine, 100 mg/l⁻ Inositol, 2 % sucrose and gelled with 7.0 % agar. Rooting was evaluated using half strength MS media supplemented with Indole-3-butyric acid (IBA), Indole-3-acetic acid (IAA), 1-naphtyleneacetic acid (NAA) and Dichlorophenoxy–acetic acid (2,4-D). Media without growth regulators was used as control. Results showed that there were significant (p<0.0001) differences among cytokinins and auxins levels for the number and length of microshoots and roots, respectively. BAP at 40 μM gave the highest mean shoot number of 15.98 ± 0.68. BAP 5 μM gave the highest mean shoot length of 1.32 ± 0.06 cm. For root induction, IBA at 10 μM gave the highest mean number (4.16 ± 1.50) of roots per explant and the highest (0.62 ± 0.02 cm) mean root length. Regenerated plantlets were successfully weaned in the greenhouse. The results from the study will enhance rapid multiplication of high quality planting materials of high pyrethrin cultivars in Rwanda. This is the first comprehensive report on the use of different cytokinins and auxins in tissue culture of pyrethrum.

Keywords: *In vitro* regeneration, Microshoot, pyrethrum, explant, Rwanda

1. Introduction

Pyrethrum (*Chrysanthemum cinerariaefolium*) is a tufty perennial herb of 30 to 100 cm in height which belongs to the Compositae family [16]. Some family members may be found to be herbaceous (including pyrethrum), but others can be shrubs, trees or climbers [11]. Pyrethrum flowers are mainly composed of pyrethrins in its flower heads and these pyrethrins are widely used as natural insecticides. Pyrethrins have some of the qualities of an ideal pest-control agent, as they are very effective against a broad range of insects, with minor development of resistant strains, and have knockdown and kill effects on insects. One of the major advantages of pyrethrins over all other insecticides is their low toxicity towards mammals and other warm-blooded animals [5]. In addition, pyrethrins are non- inflammable and leave no oily residues; because of these two reasons, it is valuable in the home where there are foodstuffs.

Rwanda is one of the pyrethrum producing countries in the Eastern African region. However, the country does not produce enough pyrethrum although there is enormous potential to expand production [14]. However, most of the pyrethrum fields are old and the plants have been found in many cases to have very low pyrethrin content. There is therefore, need to avail farmers with adequate high yielding and high pyrethrin content pyrethrum to boost production.

Pyrethrum is conventionally propagated through splits or shoots cuttings. These propagation methods results in low multiplication rates [5]. The lack of adequate, disease-free and high yielding planting materials remains the major bottleneck in pyrethrum production in Rwanda.

The first report on tissue culture of pyrethrum was by [12] reported on micropropagation of *Chrysanthemum cinerariaefolium* using flower discs as explants. The explants were cultured on MS medium supplemented with Indole-Acetic Acid (IAA), 1-naphtaleneaceticacid (NAA) at 10 μM and 6-benzyladenine at 0.1 μM. These growth regulators induced the development of plantlets after 8 weeks. The regenerated plantlets were successfully transferred to unsterilized soil where they developed roots. [15] reported on *in vitro* clonal multiplication of pyrethrum by using axillary buds as explants. The best media for regeneration was found to be MS supplemented with 0.1 mg/l BA and 1.1 mg/l NAA. The regenerated plantlets were directly potted into peat/coarse sand mixture. [6] reported that axillary bud development and shoot formation was the highest in a two-step culturing program: first shoot tips were cultured on MS

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medium supplemented with BAP (0.1μM) for 10 days and subsequently transferred to multiplication media supplemented with a higher concentration of BAP (0.9 μM) yielding on average 21.5 shoots per explant.

[4] showed that 3-benzylaminopurine at 1 mg/l induces a greater cell growth than other cytokinins while [8] standardized a protocol for multiplication of *Chrysanthemum cinerariaefolium* through somatic embryogenesis. Callus induction from leaf explants in MS medium containing 1.5 mg/l 2, 4-D was found to be 100%. The best friable calli were subjected to suspension culture in MS media supplemented with 1.0 mg/l BAP for somatic embryos. All calli in suspension gave rise to somatic embryos, which were regenerated in MS media supplemented with various concentrations of BAP. The regenerated plantlets were elongated on MS media supplemented with 0.1 mg/l BAP. Many researchers in the past have reported the use BAP of regeneration of microshoot and NAA and IAA for regeneration of roots in pyrethrum. Since *in vitro* regeneration methods are known to be genotype dependent, the current study evaluated several cytokinins and auxins to ascertain their effectiveness on plantlet regeneration in the Rwandan pyrethrum.

2. Materials and methods

2.1 Plant materials
The study was carried out at the plant tissue culture laboratory of Rwanda Agricultural Board situated in Rubona (Altitude: 1630 m asl, $2^0$29'07''S, $29^0$47'49''E), Southern Province of Rwanda. *In vitro* grown plantlets were used to provide sterile starting nodal explants for the study.

2.2 Preparation of Media
Regeneration of microshoot was carried out using full strength Murashige and Skoog (MS) basal salts [9] supplemented with 30 mg/l cysteine, 100 mg/l Inositol and 3% (w/v) sucrose. To this medium, 2iP, BAP and KIN each at 5, 10, 20 and 40 μM; TDZ at 0.1, 0.5, 1 and 1.5 μM, were added in separate experiments. On the other hand, half strength MS medium supplemented with IBA, IAA, NAA, 2,4-D each at 5, 10, 20 40 μM, 100 mg/l inositol and with 2 % sucrose was used for rooting. Medium without any growth regulator was used as the control. The pH was adjusted to 5.8 using 1 N NaOH or 0.1 M HCL before agar was added and media heated to dissolve the agar and dispensed in 10 ml aliquots into test tube. The media was autoclaved at 1.06 kg cm^{-2} and 121^{0}C for 15 min.

2.3 Inoculation and incubation
Inoculation was carried out in a sterile laminar airflow hood. Nodal cuttings were dissected from *in vitro* growing plantlets using sterile blade and forceps. Single nodal cuttings were inoculated into a test tube containing 10 ml of the sterile media under evaluation. These were incubated at 22 ± 2^0 C under a 16 h photoperiod with a photosynthetic photon flux density of 40 μmol m-2 s-1 provided by overhead cool fluorescent lamps (Philips, 30 Watts) for four weeks for regeneration of microshoot. The shoots regenerated from the nodal segments were excised and transferred to MS medium containing auxins for root initiation.

2.4 Transplanting
The *in vitro* regenerated plantlets were carefully removed from the test tubes and the roots gently cleaned with running tap water to remove agar. The plants were then carried to the green house where they were treated with 2% fungicide (red copper for 20 minutes). A weaning pot (Supplied by Sunplam Australia PTY, LTD) was filled with sterile potting mixture composed of top soil, sand and manure mixed in the ratio of 3:2:1(w/w). The pot was placed in a basin containing water to allow the potting mixture to take up water until the top became moist. The pot was then removed from the basin and the plantlets carefully planted using sharp wood sticks. After two weeks, the holes of the weaning pot were half way opened and eventually fully opened after one month. The plants were irrigated once a week for first two weeks and twice a week thereafter.

2.5 Experimental design, data collection and analysis
The experiments were laid out in completely randomized design (CRD) with twenty explants per treatment. Data on number of microshoot per explants, microshoot length, the number of roots per shoot and length of the roots were recorded on a weekly basis. All the data were subjected to one-way analysis of variance (ANOVA) and the significant differences between treatment means were assessed by least significant difference (LSD) tests at p ≤0.05 [13]. The results are expressed as a mean ± SE. Hardening of plantlets was evaluated 30 days after transfer to the greenhouse and percentage of survival plantlets was recorded.

3. Result and discussion
The effects of the different cytokinins evaluated on microshoot regeneration are shown in Table1. Of the four cytokinins evaluated BAP was more effective (P <0.0001) in inducing microshoot proliferation than all the other cytokinins. Shoot primordia on nodal explants were observed on MS medium with plant growth regulators after 2

weeks and three weeks on media without growth regulators. It was observed that when no growth regulators were incorporated in the media, only one shoot per an explant developed. Multiple shoots were induced on nodal explants cultured on media supplemented with various plant growth regulators. Browning of explants and media due to phenolic exudation has been observed as a serious problem in establishing pyrethrum cultures from field grown explants (Kahia, unpublished). However, this problem was not observed in this study where cultures were initiated from *in vitro* raised plants. This is probably because young seedlings do not synthesize phenolic substances [3].

Table 1: Effects of different cytokinins on *in vitro* regeneration of pyrethrum shoots

Regulators	Mean of microshoots	Mean length of shoots (cm)
Control	7.10 ± 0.55^e	0.72 ± 0.02^c
BAP	12.82 ± 0.62^a	1.09 ± 0.04^a
KIN	11.94 ± 0.42^b	1.02 ± 0.04^b
TDZ	10.97 ± 0.55^c	0.98 ± 0.04^b
2iP	10.19 ± 0.68^d	0.97 ± 0.05^b
P value	<.0001	0.0001
LSD	0.7567	0.0609

Values represent means $\pm$ SE. Means within a column followed by different letters are significantly different at $P = 0.05$

The effect of different concentrations of BAP on microshoot regeneration is presented in Table 2. The media supplemented with BAP at 40 µM gave the highest mean number (15.98 ± 0.68) of shoots per explant. However it was not statistically different at p=0.05 from the number of shoots obtained from BAP 5µM. The highest mean shoot length (1.34 ± 0.04) was observed on BAP at 5µM. Increasing the concentration of BAP from 5 to 20 µM resulted in a decrease in the mean number of shoots and the mean shoot length. However, when the concentration of BAP was increased from 20 to 40 µM, there was an increase in the mean number of shoots per explant. The control had the lowest mean number of shoots. The results of the current study are in agreement with those of [10] who reported that shoot proliferation from pyrethrum nodal culture was better on a media supplemented with BAP than with kinetin. Superiority of BAP for shoot induction may be attributed to the ability of plant tissues to metabolize BAP more readily than other synthetic growth regulators or to the ability of BAP to induce production of natural hormones such as zeatin, within the tissue [7].

Table 2: Effects of different BAP concentrations on microshoot proliferation and growth

Concentration (µM)	Mean of microshoots	Mean length of shoots(cm)
Control	1.10 ± 0.55^c	0.72 ± 0.02^d
5	14.93 ± 0.6^a	1.34 ± 0.04^a
10	9.61 ± 0.42^b	0.93 ± 0.04^c
20	10.76 ± 0.55^b	0.95 ± 0.04^c
40	15.98 ± 0.68^a	1.16 ± 0.05^b
P value	<.0001	<.0001
LSD	1.5984	0.1158

Values represent means $\pm$ SE. Means within a column followed by different letters are significantly different at $P = 0.05$

The effect of kinetin on microshoot regeneration is presented in Table 3. The medium supplemented with kinetin 40 µM gave the highest mean number (14.33 ± 0.66) of microshoots per explant and the highest mean length (1.12 ± 0.04) of shoots. Increasing the concentration of kinetin from 5 to 10 µM resulted in a slight increase in the mean number of shoots per explant only and their respective lengths. However an increase in the concentration of kinetin from 10 to 40 µM resulted in a significant increase in the mean number of shoots per explant and the mean length of shoots. The control had the lowest mean shoot number and mean shoot length. The performance of kinetin at the higher concentration of 40 µM was comparable to that of the best BAP concentration.

Table 3: Effects of different kinetin concentrations on microshoot proliferation and growth

Concentration (µM)	Mean of microshoots	Mean length of shoots (cm)
Control	1.10 ± 0.55^c	0.72 ± 0.02^b

5	9.63 ± 0.72^{b}	0.83 ± 0.06^{b}
10	10.07 ± 0.55^{b}	1.02 ± 0.04^{a}
20	13.74 ± 0.50^{a}	1.11 ± 0.05^{a}
40	14.33 ± 0.66^{a}	1.12 ± 0.04^{a}
P value	<.0001	<.0001
LSD	1.7086	0.134

Values represent means ± SE. Means within a column followed by different letters are significantly different at $P = 0.05$.

The medium supplemented with TDZ at 0.5 µM gave the highest mean number (11.52±0.46) of shoots per explant and length (1.0±0.04) (Table 4). However, these values were not significantly different from those obtained with 0.1 µM TDZ. Increasing the concentration of TDZ from 0.1 to 0.5 µM resulted in a slight increase in the mean number of shoots per explant and the mean length of shoots. There was no significant difference in the mean shoot length in all the TDZ concentrations evaluated. This is the first report on the use of TDZ in regeneration of pyrethrum microshoots. These results are in agreement to those of [1] while working on bananas that showed that shoot proliferation progressively increased with increasing TDZ concentrations up to 1 mg/l.

Table 4: Effects of different TDZ concentrations on microshoot proliferation and growth

Concentration (µM)	Mean of microshoots	Mean length of shoots (cm)
Control	1.10 ± 0.55^{c}	0.72 ± 0.02^{b}
0.5	11.52 ± 0.46^{a}	1.0 ± 0.04^{a}
0.1	11.06 ± 0.44^{ba}	0.94 ± 0.04^{a}
1.0	10.24 ± 0.39^{b}	0.94 ± 0.03^{a}
1.5	11.07 ± 0.45^{ba}	0.97 ± 0.04^{a}
P value	0.0114	0.0318
LSD	1.2058	0.1057

Values represent means ± SE. Means within a column followed by different letters are significantly different at $P = 0.05$.

The effects of 2iP on microshoot regeneration are presented in Table 5. 2iP at a concentration of 20 µM gave the highest mean number of shoots (11.30±0.73) although this was not significantly different from the numbers obtained at a concentration of 5 or 40 µM. On the other hand, the highest mean length of shoots (1.16±0.05) was observed on a media supplemented with 2iP at 5 µM.

Table 5: Effects of different 2iP concentrations on microshoot proliferation and growth

Concentration (µM)	Mean of microshoot	Mean length of shoots (cm)
Control	1.10 ± 0.55^{c}	0.72 ± 0.02^{c}
5	10.19 ± 0.42^{ba}	1.16 ± 0.05^{a}
10	9.15 ± 0.43^{b}	0.97 ± 0.03^{b}
20	11.30 ± 0.73^{a}	0.83 ± 0.06^{c}
40	11.13 ± 0.54^{a}	0.94 ± 0.04^{cb}
P value	0.0471	<.0001
LSD	1.5206	0.1316

Values represent means ± SE. Means within a column followed by different letters are significantly different at $P = 0.05$

Among all the auxins evaluated, roots were only induced in media supplemented with IBA and NAA (Table 6). The effects of the various concentrations of IBA on induction of rooting on pyrethrum microshoots are presented in Table 7. The lower concentration (5µM) of IBA did not produce any roots. On the other hand, IBA at 10 µM gave the highest mean number (4.17 ± 0.51) of roots per explant and the highest mean length (0.63 ± 0.02) of root per explant (Plate 2). Increasing the concentration of IBA from 5 to 10 µM resulted in a significant increase in the mean number and mean length of shoots. However, increasing the concentration of IBA from 10 to 20 µM resulted in a significant decrease in the number of roots per microshoot. The microshoots cultured on media supplemented with NAA 10 µM gave the

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highest mean number (1.17±0.64) of roots and the mean length (0.17±0.09); all other concentrations evaluated did not induce any roots (Table 8).

Table 6: Effects of different auxins on *in vitro* rooting of pyrethrum microshoots

Auxin type	Mean of roots	Mean length of roots (cm)
Control	0.0 ± 0.0^b	$0.0 \quad 0.0^b$
IAA	0.0 ± 0.0^b	$0.0 \quad 0.0^b$
IBA	2.60 ± 0.31^a	0.36 ± 0.04^a
2,4 D	0.0 ± 0.0^b	0.0 ± 0.0^b
NAA	0.29 ± 0.17^b	0.04 ± 0.02^b
P value	<.0001	0.0001
LSD	0.354	0.0413

Values represent means ± SE. Means within a column followed by different letters are significantly different at *P* = 0.05.

Table 7: Effects of different IBA concentrations on root induction on pyrethrum microshoots

Concentration (µM)	Mean of roots	Mean length of shoots
Control	0.0 ± 0.0^c	0.0 ± 0.0^c
5	0.0 ± 0.0^c	0.0 ± 0.0^c
10	4.17 ± 0.51^a	0.63 ± 0.02^a
20	2.42 ± 0.51^b	0.38 ± 0.07^b
40	3.83 ± 0.32^a	0.45 ± 0.03^b
P value	<.0001	<.0001
LSD	1.1249	0.1136

Values represent means ± SE. Means within a column followed by different letters are significantly different at *P* = 0.05.

Table 8: Effects of different NAA concentrations on root induction on pyrethrum microshoots

Concentration (µM)	Mean of roots	Mean length of shoots
Control	0.0 ± 0.0^b	0.0 ± 0.0^b
5	0.0 ± 0.0^b	0.0 ± 0.0^b
10	1.17 ± 0.64^a	0.17 ± 0.09^a
20	0.0 ± 0.0^b	0.0 ± 0.0^b
40	0.0 ± 0.0^b	0.0 ± 0.0^b
P value	0.0274	0.0197
LSD	0.9087	0.1245

Values represent means ± SE. Means within a column followed by different letters are significantly different at *P* = 0.05
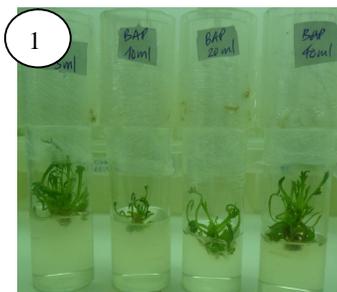

Plate 1: Microshoots regeneration from nodal explants. (From Left to Right: BAP at 5, 10, 20 and 40 µM).
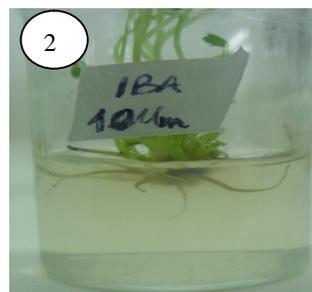

Plate 2: Rooting of regenerated microshoots on a medium supplemented with IBA at 10 µM.

Addition of auxins in the media usually favours the rooting phase, although the response varies with concentrations [2]. IBA at 10 µM was found to be the best growth regulator for regenerating roots from pyrethrum microshoots during the

current study. These results are contrary to those of [10] who reported that NAA was superior in rooting of pyrethrum microshoots than IBA. However, it was in agreement with the results of [16] who reported that IBA gave better results of root induction and development from pyrethrum microshoots than all the concentrations of NAA evaluated. This is the first comprehensive report on evaluation of several cytokinins and auxins on regeneration of pyrethrum plantlets. *In vitro* regenerated pyrethrum plantlets were acclimatized in the greenhouse conditions after placing in pots (Plate 3) containing soil sand and manure mixture (3:2:1). After acclimatization for one month, about 60% survival rate was recorded (Data not shown).
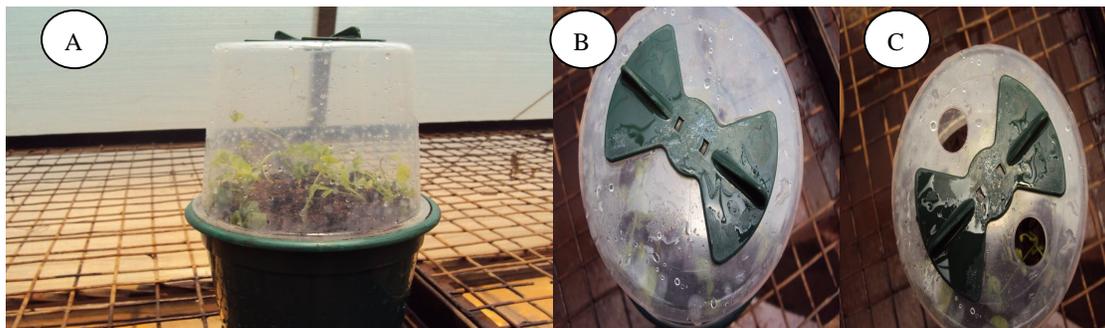

Plate 3: A Weaning pot; **B**: the top showing closed holes which are opened after two weeks
C: the opening on top of the weaning pot

During the current study, a reproducible protocol of regenerating pyrethrum plantlets was developed using *in vitro* raised nodal explants. The protocol involves culturing nodal explant in MS media supplemented with 5 µM BAP, 30 mg / l cysteine, 100 mg/ l Inositol and 3% (w/v) sucrose for four weeks to regenerate microshoots. The microshoots are rooted on Half strength MS media supplemented with 10 µM IBA, 30 mg / l cysteine, 100 mg/ l Inositol and 2% (w/v) sucrose for one month. The results of the study will enhance rapid production of pyrethrum planting materials and open new prospects for massive propagation of the Rwandan high pyrethrin content varieties, which are highly needed to replace the genetically eroded clones in the country.

4. Conclusion
An efficient protocol was developed for *in vitro* propagation of the elite Rwandan pyrethrum cultivar *using* nodal explants obtained from *in vitro* raised plantlets of pyrethrum was developed during the current study. However, there is a need to evaluate the performance of the regenerated plantlets in the field.

Acknowledgment

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