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IN VITRO REGENARATIONOF ARTEMISIA ANNUA (WORMWOOD) USING SEED EXPLANTS

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ABSTRACT

The most effective concentration of some plant growth hormones on the in vitro regeneration of Artemisia annua using seed explants was investigated in the Biotechnology Laboratory of Plant Science Department of Ahmadu Bello University, Zaria. Fresh and healthy seeds of a Chinyong variety were sourced, sterilized and inoculated on a full and half strengths Murashige & Skoog basal media supplemented with varying concentrations of plant growth hormones using the procedure of Hamish, 1998. Combined treatment of GA3 (1.0 µm/l) and NAA (0.5 µm/l) in a half strength MS media recorded the fewer days to germination. Highest germination percentage was observed at combination of GA3 (2.0 µm/l) and BAP (0.5 µm/l) in a full strength MS media. Equal concentration of combined GA3 and BAP (ie 0.5 µm/l) in a full strength MS media had the best vigor, followed by varying concentration of GA3 (2.5 µm/l) and NAA (0.5 µm/l) in a half strength MS media. Highest seedling height was observed at equal concentration of combined GA3 and BAP (ie 0.5 µm/l) in a full strength MS media. This was followed by varying concentration of GA3 (0.5 µm/l) and NAA (1.0 µm/l) in a full strength MS media. Result of Analysis of Variance indicated significant difference among some of the treatments compared with the control (P≤0.05). Treating Artemisia seeds using these plant growth hormones had reduced the effect of the phenolic secretions reported on the seeds thereby enhancing its germination. Therefore, this is a promising approach to faster in vitro regeneration of Artemisia plant.

Keywords: Artemisia, In vitro, Germination, Plant growth hormones.

1. INTRODUCTION

Artemisia (wormwood) belongs to the family Asteraceae (Compositae) and consist of about 400 species ($2n = 36$). It is an annual medicinal herb native to China and it is one of the few species in this genera in which essential oils, aromatic wreaths and an anti malarial agent (Artemisinin) has been detected and isolated.(Bailey and Bailey, 1976; Bennett *et al*., 1982;

McVaugh, 1984; Elhaq *et al*., 1991; Klayman, 1993; Jaime and da silva, 2003).The family is characterized by extreme bitterness of all parts of the plant(Tripathi *et al*., 2000; Tripathi *et al*., 2001; Ferreira and Janck, 2009). Its cultivation has expanded in China and Africa, mainly Kenya, Tanzania and Nigeria in response to the call by the World Health Organization for the use of Artemisinin-Combination Therapies (ACT). (Ferreira *et al*., 2005; Brisibe, 2006).Likewise its effectiveness has been demonstrated in the treatment of skin diseases and it has also been shown to be an effective non-selective herbicide such as glyphosate.(Duke *et al*., 1987; Paniego and Giulietti, 1994). Members of some plant families exhibit erratic germination due to seed dormancy.(Bewley and Michael, 1994). A seed of *Artemisia* weighted 0.03g (World Health Organization(WHO), 2006). The seeds were observed to undergo chemical dormancy due to the Presence of some chemical compounds (such as Phenolics) on the surface. This was linked with seeds germination inhibition and dormancy of the plant.Phenolics accumulation played a protective role in strengthening the plant cell walls during growth by polymerization into lignin (Farouk *et al*., 2008). In Nigeria, productive A*rtemisia* seed is expensive and not readily available. Massive production of *A. annua* is an important step towards maximizing Artemisinin supply. However, this has been hindered by several biotic and abiotic factors such as the pest, diseases and climatic constraint, conservative agricultural practices and devastating decline in the natural habitats which expose them to the threshold of extinction(Hamish and Sue, 1998; Trigiano and Gray, 2000).. Consequently, this has led to poor performance, hence decrease in yields. These constraints may be overcome through the use of *in vitro* facilities using tissue culture technique(Trigiano and Gray, 2000).This project was designed to determine the most effective treatment for the propagation of Artemisia using seed explants.

2. MATERIALS AND METHODS

2.1. Materials

Fresh and healthy seed explants of a Chinyong variety were sourced, sterilized and inoculated on a Murashige and Skoog (1962) basal media supplemented with varying concentrations of growth hormones using the procedure of (Hamish and Sue, 1998)

2.2. Study Area

The experiment was conducted in the Biotechnology Laboratory of Plant Science Department, Ahmadu Bello University, Zaria, latitude 11 º 11! N and 07º38! E, altitude 670 m above mean sea level, 640 km from the Atlantic shores of Nigeria in the south.

2.3. Parameters Studied

The following parameters were studied:

i. Days to germination
ii. % germination
iii. Vigor

iv. Plantlet height

2.4. Treatments

Fresh and healthy seeds *of A. annua*were sourced from the Artemisia Programme Unit of Institute for Agricultural Research (IAR) ABU, Zaria. 349.2g of seeds were weighted and surface sterilized in an autoclaved cloth using 90% ethanol for 5 minutes followed by disinfection with 1% Mercuric Chloride(MgCl) for 4 minutes. Seeds were then rinsed five times in sterile distilled water before inoculation on media.

Full and half strengths Murashige and Skoog (1962) containing 9g sucrose, 30 mg Myoinositol augmented with varying concentrations of GA3, NAA and BAP was prepared. (Table I) Hormone free MS medium served as control. The pH of the medium was adjusted to 5.8±1 before the addition of 3g agar. 50ml of the media were dispensed into tubes and autoclaved at 121°C at 15 lb pressure for 15 min. The seeds were inoculated aseptically and the cultures were maintained at 24 ± 1°C using 14/10 light/dark period, under a light intensity of 3000 lux provided by cool-white fluorescent lamps and 50 to 60% relative humidity.

Table-1. Hormone composition for the regeneration of Seed explants in Full & Half strengths MS Media

Hormone	**Concentration (($\mu m/l$)**											
GA3/BAP												
GA3	0.5	1.0	1.5	2.0	2.5	3.0	0.5	1.0	1.5	2.0	2.5	3.0
BAP	0.5	0.5	0.5	0.5	0.5	0.5	1.0	1.0	1.0	1.0	1.0	1.0
BAP/NAA												
BAP	0.5	0.5	0.5	0.5	0.5	0.5	1.0	1.0	1.0	1.0	1.0	1.0
NAA	0.5	1.0	1.5	2.0	2.5	3.0	0.5	1.0	1.5	2.0	2.5	3.0
GA3/NAA												
GA3	0.5	1.0	1.5	2.0	2.5	3.0	0.5	1.0	1.5	2.0	2.5	3.0
NAA	0.5	0.5	0.5	0.5	0.5	0.5	1.0	1.0	1.0	1.0	1.0	1.0
control	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0

2.5. Analysis of Data

The data was analyzed using Analysis of Variance (ANOVA), SAS statistical package. T-tests was also used to compare treatment means and determine the Least Significant Difference ($P < 0.05$).

3. RESULTS

The addition of plant growth hormones had significant effect on the *in vitro* cultures of *A. annua* compared with the hormone free MS (Control) media which did not show any response. However, combined treatment of GA3 (1.0 µm/l) and NAA (0.5 µm/l) in a half strength MS

media recorded the fewer days to germination(Table V). However, result of analysis of variance showed no significant difference compared with combination of GA3 (1.5 µm/l) and BAP (0.5 µm/l) in a half strength MS media(Table VI). Highest days to germination was observed at combined GA3 (2.5 µm/l) and BAP (1.0 µm/l) treatment in a full strength MS media (Table II).

Highest germination percentage was observed at combination of GA3 (2.0 µm/l) and BAP (0.5 µm/l) in a full strength MS media (Table II). It was followed by GA3 (2.5 µm/l) and NAA (0.5 µm/l) in a half strength MS media(Table V). Equal concentration of combined GA3 and BAP (ie 0.5 µm/l) in a full strength MS media had the best vigor (Table II), followed by varying concentration of GA3 (2.5 µm/l) and NAA (0.5 µm/l) in a half strength MS media(Table V). Highest seedling height was observed at equal concentration of combined GA3 and BAP (ie 0.5 µm/l) in a full strength MS media(Table II). This was followed by varying concentration of GA3 (0.5 µm/l) and NAA (1.0 µm/l) in a full strength MS media (Table III).

Table- 2. Response of *A. annua*seeds to GA3 & BAP in a Full strengths MS Media

Treatment $(\mu m/l)$	**Vigor**	**Shoot length(CM)**	**Days to Germination**	**GerminationPercent (%)**
GA3 0.5 BAP 0.5	1.50ab	3.83a	11.50a	30.00ab
GA3 1.0 BAP 0.5	3.25a	2.00b	10.50ab	4.00ab
GA3 1.5 BAP 0.5	0.00b	0.00c	0.00b	0.00b
GA3 2.0 BAP 0.5	2.58a	2.25b	7.00ab	46.50a
GA3 2.5 BAP 0.5	2.83a	2.25b	10.00ab	5.40ab
GA3 3.0 BAP 0.5	1.79ab	2.42b	9.50ab	1.25ab
GA3 0.5 BAP 1.0	3.00a	2.50b	16.00a	0.40b
GA3 1.0 BAP 1.0	0.00b	0.00c	0.00b	0.00b
GA3 1.5 BAP 1.0	0.00b	0.00c	0.00b	0.00b
GA3 2.0 BAP 1.0	3.17a	2.17b	17.00a	6.50ab
GA3 2.5 BAP 1.0	2.45a	2.50b	8.50ab	2.50b
GA3 3.0 BAP 1.0	3.00a	2.00b	8.50ab	0.40b
Control	0.0b	0.0c	0.0b	0.0b

Means within a column followed by the same letter are not significantly different (p=0.05)

Table-3. Response of A. annuaseeds to GA3 & NAA in a full strengths MS Media

Treatment ($\mu m/l$)	Vigor	Shoot length(CM)	Days to Germination	GerminationPercent (%)
GA3 0.5 NAA 0.5	0.00a	0.00b	0.00a	0.00a
GA3 1.0 NAA 0.5	0.00a	0.00b	0.00a	0.00a
GA3 1.5 NAA 0.5	3.50a	2.42a	5.00a	0.03a
GA3 2.0 NAA 0.5	0.00a	0.00b	0.00a	0.00a
GA3 2.5 NAA 0.5	0.00a	0.00b	0.00a	0.00a
GA3 3.0 NAA 0.5	3.50a	3.00a	10.00a	1.50a
GA3 0.5 NAA 1.0	3.00a	3.15a	6.00a	7.50a
GA3 1.0 NAA 1.0	0.00a	0.00b	0.00a	0.00a
GA3 1.5 NAA 1.0	2.51a	2.25a	5.50a	8.50a
GA3 2.0 NAA 1.0	0.00a	0.00b	0.00a	0.00a
GA3 2.5 NAA 1.0	0.00a	0.00b	0.00a	0.00a
GA3 3.0 NAA 1.0	0.00a	0.00b	0.00a	0.00a
Control	0.0a	0.0b	0.0a	0.0a

Means within a column followed by the same letter are not significantly different (p=0.05)

Table- 4. Response of A. annuaseeds to BAP & NAA in a Full strengths MS Media

Treatment ($\mu m/l$)	Vigor	Shoot length(CM)	Days to Germination	Germination Percent (%)
BAP 0.5 NAA 0.5	0.00b	0.00b	0.00a	0.00b
BAP 0.5 NAA 1.0	3.08a	2.67a	6.00a	4.00a
BAP 0.5 NAA 1.5	3.00a	2.50a	10.00a	1.25ab
BAP 0.5 NAA 2.0	0.00b	0.00b	0.00a	0.00b
BAP 0.5 NAA 2.5	0.00b	0.00b	0.00a	0.00b
BAP 0.5 NAA 3.0	0.00b	0.00b	0.00a	0.00b
BAP 1.0 NAA 0.5	0.00b	0.00b	0.00a	0.00b
BAP 1.0 NAA 1.0	0.00b	0.00b	0.00a	0.00b
BAP 1.0 NAA 1.5	0.00b	0.00b	0.00a	0.00b

BAP 1.0 NAA 2.0	0.00b	0.00b	0.00a	0.00b
BAP 1.0 NAA 2.5	0.00b	0.00b	0.00a	0.00b
BAP 1.0 NAA 3.0	0.00b	0.00b	0.00a	0.00b
Control	0.0b	0.0b	0.0a	0.0b

Means within a column followed by the same letter are not significantly different (p=0.05)

Table-5. Response of A. annuaseeds to GA3 & NAA in a Half strengths MS Media

Treatment ($\mu m/l$)	Vigor	Shoot length(CM)	Days to Germination	Germination Percent (%)
GA3 0.5 NAA 0.5	2.67abc	2.17abc	2.00ab	0.20e
GA3 1.0 NAA 0.5	3.00abc	2.00bcd	1.50bc	22.50a
GA3 1.5 NAA 0.5	3.17ab	2.08bc	3.00ab	19.50a
GA3 2.0 NAA 0.5	2.42bcd	2.08bc	3.00ab	11.25ab
GA3 2.5 NAA 0.5	1.67d	2.75a	1.50bc	33.50a
GA 3 3.0 NAA 0.5	2.25dc	2.58ab	6.00ab	27.50a
GA3 0.5 NAA 0.5	3.42a	1.58dc	3.00ab	5.00bc
GA3 1.0 NAA 1.0	0.00e	0.00e	0.00e	0.00e
GA3 1.5 NAA 1.0	3.33a	1.42d	10.00a	2.00cd
GA3 2.0 NAA 1.0	0.00e	0.00e	0.00e	0.00e
GA3 2.5 NAA 1.0	3.42a	1.58dc	3.00ab	2.50cd
GA3 3.0 NAA 1.0	3.17b	1.58dc	7.00ab	5.00bc
Control	0.0e	0.0e	0.0e	0.0e

Means within a column followed by the same letter are not significantly different (p=0.05)

Table- 6. Response of A. annuaseeds to GA3 & BAP in a half strengths MS Media

Treatment ($\mu m/l$)	Vigor	Shoot length(CM)	Days to Germination	Germination Percent (%)
GA3 0.5 BAP 0.5	2.75a	1.35b	12.50a	1.25a
GA3 1.0 BAP 0.5	0.00b	0.00c	0.00b	0.00a
GA3 1.5 BAP 0.5	2.99a	2.10a	1.50ab	12.00a
GA3 2.0	0.00b	0.00c	0.00b	0.00a

BAP 0.5				
GA3 2.5 BAP 0.5	2.88a	2.00a	6.00ab	16.50a
GA3 3.0 BAP 0.5	0.00b	0.00c	0.00b	0.00a
GA3 0.5 BAP 1.0	0.00b	0.00c	0.00b	0.00a
GA3 1.0 BAP 1.0	0.00b	0.00c	0.00b	0.00a
GA3 1.5 BAP 1.0	0.00b	0.00c	0.00b	0.00a
GA3 2.0 BAP 1.0	0.00b	0.00c	0.00b	0.00a
GA3 2.5 BAP 1.0	0.00b	0.00c	0.00b	0.00a
GA3 3.0 BAP 1.0	0.00b	0.00c	0.00b	0.00a
Control	0.0b	0.0c	0.0b	0.0a

Means within a column followed by the same letter are not significantly different (p=0.05)

Fig- 1. Early In vitro seed germination

Fig-2. Later stage of In vitro germination

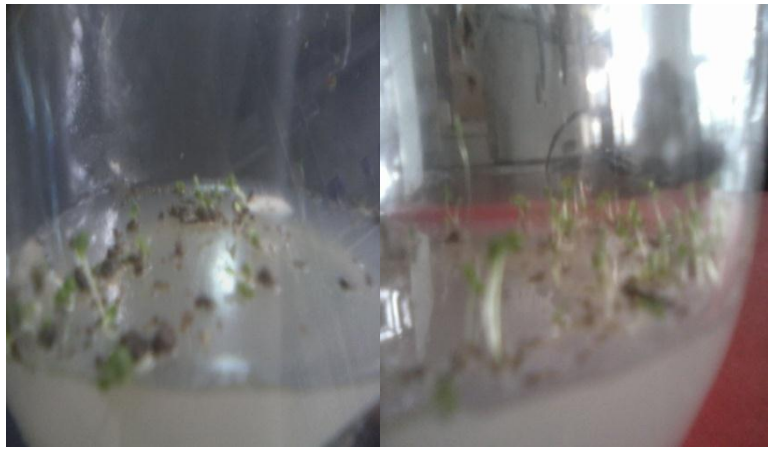
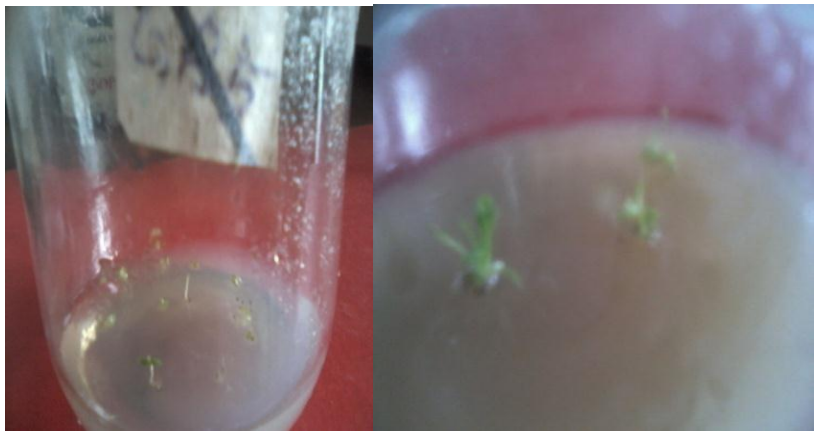


Fig- 3. In vitro Plantlets responding to treatment

Fig- 4. Plantlets sub-cultured into a fresh MS Media.



4. DISCUSSION

This experiment was based on the principle of manipulation of the MS media and the hormonal concentrations (Murashige, 1990; Uranbey *et al*., 2005) ((Bewley and Michael, 1994) The germinating seeds of *A. annua* exhibited a hypogeal type of germination by having the Cotyledon remaining below the media surface. A seed was considered germinated when the tip of the radicle had grown free of the seed coat emerging through the outer covering (Wiese and Binning, 1987; Auld *et al*., 1988). Exposure of the shoot tip to light enabled it to photosynthesize thereby straightening the epicotyls. (Moore, 1979; Osborne *et al*., 1985)

Chemical dormancy reported due to phenolic secretion on the seed of *A. annua*seeds was overcome by the use of GA3 (Bewley and Michael, 1994; Nicolas, 2003; Farouk *et al*., 2008). Similarly, the cytokinin (BAP) had positively influenced the physiological process of the seed germination as reported by (Thomas, 1989; Syed, 2001) it also enhances lateral bud growth. All seeds responded to treatments when kept under the light. This is contrary to the findings of (Jamaleddine *et al*., 2011)who reported that seeds of *A. annua* germinated after exposure to dark. Germination of seeds of *A. annua*, commenced 3 days after inoculation, and 50 to 60% of the

seeds regenerated to plantlets. This is contrary to the findings of (Jamaleddine *et al*., 2011) and (Mannan *et al*., 2012) and their coworkers who observed that plant growth regulators do not reduce the number of days to germination in the *ln vitro* cultures of *A. annua* and *A.absinthium*compared to 6 - 7 days obtainable when grown under field conditions. Also, no increase in the germination percentage was observed. This is not in conformity with the results obtained by (Nikolić *et al*., 2006) working on seeds of *Lotus coniculatus* who reported that different hormones and their optimum concentrations stimulated germination percent at least two fold. No multiple shoots were recorded from this experiment as also reported by (Jamaleddine *et al*., 2011). Similarly, the addition of GA3, auxin and cytokinin had significantly shorten the number of days to germination, vigor and shoot length. Mark Christopher McCoy (2003) had also suggested that the hormones GA3, BAP, and NAA stimulates growth in *A.annua.*

Half strength MS media with a combined treatment of GA3 (1.0 µm/l) and NAA (0.5 µm/l) was the most suitable for *in vitro* germination of *A.annua* seeds.

5. CONCLUSION

In vitro propagation and multiplication of A. annua plant resources through seed culture is a promising technique for producing disease and contamination free plantlets, especially that it's seeds were observed to undergo a long dormancy period and they are scarce and expensive.

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