

In Vitro Germination and Plantlet Establishment of Wild Chamomile of Morocco *Cladanthus mixtus* (L.) Oberpr. and Vogt

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Abstract

In vitro seeds germination and plantlets establishment of *Cladanthus mixtus* (L.) Oberpr. and Vogt were studied in this report. A reliable protocol was developed for *in vitro* seed germination, multiplication and plantlet regeneration of *Cladanthus mixtus*. The seeds were sterilized and cultured in different media. Among the two basal media evaluated for symbiotic seed germination, gelled distilled water was found to be the best with a high percentage of seeds germination (100%) after 33 hours of culture. Seedlings were further transferred to different types of media. Result observed after 4 weeks showed that MS medium promotes the highest growth with an average of 2.75 ± 0.12 cm shoot length and 2.60 ± 0.29 shoots per explants, and the mean number of roots achieved 3.33 ± 0.17 root per explants with a length of 2.42 ± 0.16 cm. This study showed that macroelements of MS (1962) medium is essential for *in vitro* shoot multiplication, growth and rooting of shoots of *Cladanthus mixtus* L.

Keywords

Asymbiotic Seed Germination, *In Vitro* Propagation, Basal Media, *Cladanthus mixtus* (L.)

1. Introduction

Tribe Anthémidées includes 109 genus and 1740 species [1]. The phylogenetic studies have confused the names

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of species Anthemideae, including genus *Chamaemelum*, *Ormenis* and *Cladanthus* [2].

Genus *Cladanthus* is clearly related to the genera *Anthemis* and *Chamaemelum* and most species were previously included in the last genus. However, a molecular study of *Chamaemelum* Mill. and related genus showed that traditionally marked as *Chamaemelum* is paraphyletic [2].

Cladanthus is a small genus of five species, all native to the Mediterranean region and south-west Europe [3].

Cladanthus mixtus (L.) Oberpr. and Vogt, wild Moroccan chamomile, is an aromatic plant known as the vernacular name “Hellâla” [4], and the newly adopted scientific, including the synonyms *Anthemis mixta* L., *Chamomilla mixta* Grenier et Godron, *Chamaemelum mixtum* (L.) All., *Chamaemelum mixtum* (L.) All. var. *mixtum*, *Chamaemelum mixtum* var. *aureum* (Durieu) Benedi, *Chamaemelum mixtum* var. *glabrescens* (Maire) Benedi, *Ormenis aurea* Durieu, *Ormenis mixta* (L.) Dumort. subsp. *mixta*, *Ormenis mixta* subsp. *aurea* (Durieu) Batt., *Ormenis mixta* subsp. *multicaulis* (Braun-Blanq. & Maire) Maire, *Ormenis mixta* var. *glabrescens* Maire [5].

The Moroccan chamomile, or simple-leaved chamomile, is a tall spontaneous annual plant, fragrant, 1 m in height, with the peripheral ligulate flowers which are white, a golden yellow at the base and the central florets are tubular; achenes are all identical, devoid of pappus three coasts visible on the internal face, smooth outside [6] [7].

Moroccan Chamomile widespread in Algeria, Morocco, northern and eastern part of the Mediterranean basin [7], grows in sandy soils in northern Morocco along the Atlantic coast [8]. It is usually found in woodlands, fields and pastures sandy and stony plain and low mountains [9].

The infusion of Moroccan chamomile leaves and flowers is used in Moroccan traditional medicine for treatment of different ailments [4] [10].

Moroccan Chamomile is mainly used for the extraction of essential oil of camphor odor; Morocco is the leading provider in the international market; it is sought in cosmetics, medicine and especially in perfumery [11]-[14].

In Morocco, wild Chamomile recommended as an anxiolytic and rebalancing the central nervous system, is recommended in nervous breakdowns, for shortcomings liver and stomach light and colibacillaires colitis [12].

The essential oil of *Cladanthus mixtus* is active *in vitro* against *Escherichia coli*, *Bacillus subtilis*, *Staphylococcus aureus* and *Micrococcus luteus* and fungi *Penicillium parasiticus*, *Aspergillus niger* and *Trametes pini* [15].

Extract of *Cladanthus mixtus* inhibits the corrosion of steel in acidic medium. A critical concentration of extract provides the maximum protection [16].

The plants used in the phyto-pharmaceutical preparations are obtained mainly from the natural growing areas. With the increase in the demand for the extract of these plants, the plants are being overexploited, threatening the survival of many species. Also, many medicinal plant species are disappearing at an alarming rate due to rapid agricultural and urban development, uncontrolled deforestation, and indiscriminate collection. Advanced biotechnological methods of culturing plant cells and tissues should provide new means for conserving and rapidly propagating valuable, rare, and endangered medicinal plants.

Although *Cladanthus mixtus* presents a significant medicinal and aromatic value, no study focuses on the micropropagation of this species; *in vitro* culture is the subject of a new study. So we are interested in the vegetative propagation by *in vitro* culture (micropropagation) of chamomile Morocco: *C. mixtus* L.

The aim of this paper is to test the effect of different culture media on *in vitro* seed germination of *Cladanthus mixtus* (L.), and micropropagation, multiplication and rooting of seedling germination.

2. Materials and Methods

2.1. Plant Material and Sterilization Method

Achenes of *Cladanthus mixtus* (L.) were provided by INRA Kenitra (Morocco) under the name *Ormenis mixta* (L.) Dumort. They were collected from the Maamoura (Morocco) in 2007.

Achenes are sorted and cleared the rest of the capitulum. They are put in transparent plastic bags and stocked in the dark at room temperature.

Sterilization was performed according to the following protocol: The seeds were submerged in 7% (v/v) calcium hypochlorite for 15 min with additional of three drops of Tween 20, then immersed in 0.1% (v/v) mercuric chloride (HgCl₂) for 1 min. and finally rinsed three times (5, 10 and 15 min) with sterile distilled water to remove traces of chlorine.

2.2. Effect of Basal Media on *in Vitro* Seed Germination

The sterilized seeds were inoculated into sterilized culture into Petri dishes (9 mm) containing 20 ml of different basal media. Thirty seeds were incubated for each treatment and repeated three times, and the seedlings used in subsequent experiments.

The basal media screened, to identify the suitable medium for seed germination were: Gautheret (1959) [17] (Table 1) and gelled distilled water.

All media were supplemented with 3% sucrose and gelled with 0.7% agar Bacteriological and adjusted to a pH of 5.6 - 5.8 with NaOH and HCl prior to autoclaving at 120°C for 20 min. All the cultures were incubated at 25°C under white fluorescent light intensity (800 lux) with a 16 hours photoperiod and 80% relative humidity.

The percentage (%) of seeds germination was visually monitored on each hour, and it was recorded in Figure 1. The seeds considered to be germinated by the emergence of radicles from the seeds [18]. Percentage of seed germination was calculated according to the following equation (adapted from [19]):

$$\% \text{ seed germination} = \frac{\text{Number of seeds germinated}}{\text{Total number of seeds}} \times 100$$

2.3. Effect of Basic Culture Media on Plantlet Development from Shoot Tips from *Cladanthus mixtus* (L.)

The young seedlings used of 2 - 3 mm were obtained from *in vitro* germination of seeds (2 leaf stage cotyledonary) of 100 seedlings. They are disposed vertically to the surface of a culture medium containing the macroelements, microelements and vitamins of Murashige and Skoog (1962), myo-inositol (meso-inositol) 100 mg/l, 3% sucrose (Table 2) and solidified with 0.7% bacteriological agar type E.

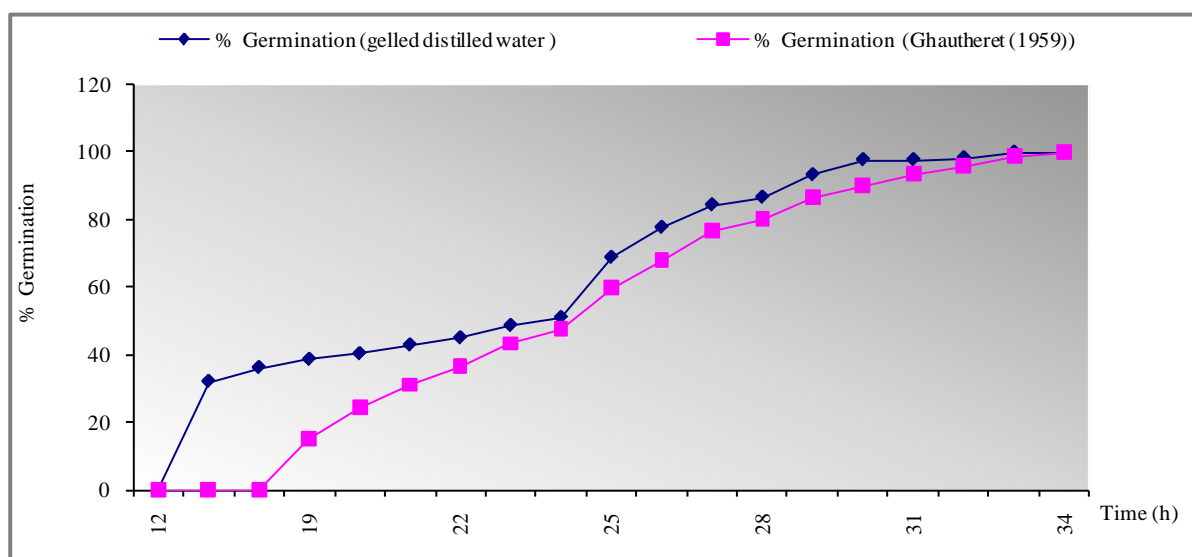


Figure 1. Effect of medium composition on the germination of seeds *Cladanthus mixtus* L.

Table 1. Macroelements of Gautheret (1959) used during germination of seeds of *Cladanthus mixtus* L.

Macroelements	mg/l	mM
KNO ₃	125	1.24
MgSO ₄ ·7H ₂ O	125	0.51
KH ₂ PO ₄	125	0.92
Ca(NO ₃) ₂ ·4H ₂ O	500	2.12

Table 2. Vitamins and sugars MS medium (1962).

Vitamins	mg/l	mM
Glycine	2.00	26.64
Thiamine-HCl	0.10	0.30
Acide nicotinique	0.50	4.06
Pyridoxine-HCl	0.50	2.43
Sugars	mg/l	mM
Myo-Inositol	100	555
Sucrose	3000	8.76×10^4

Transplanting is done every 30 days, three clones A (small size), B (medium size) and C (long size) were selected from the 9th transplant. The development of the culture medium was carried out on the clone C (long size).

To determine the most favorable to the growth of explants nutrient conditions we chose settings the salt solutions of MS: Murashige and Skoog (1962) [20], SD: Sahah and Dalal (1978) [21], SH: Schenk and Hildebrandt (1972) [22], MSm: Murashige and Skoog modified (Badoc, 1982) [23], and B₅: Gamborg and Eveleigh (1968) [24], (Table 3), with microelements and vitamins of Murashige and Skoog (1962), myo-inositol (meso-inositol) 100 mg/l, agar (0.7%) and 3% sucrose, were tested on the apex of *cladanthus mixtus* L. (clone A).

These macronutrients are different by their nitrogen (NO_3^- and NH_4^+) and potassium content (Table 4). After 30 days in culture the response was evaluated. The results presented in Table 5, and with examples in Figure 2 and Figure 3, Numbers of shoots, root as well as shoot height and root length, percentage of hyperhydricity and callogenesis were determined after 4 week of culture.

2.4. Statistical Analysis

Thirty explants were used per treatment and the experiment was repeated twice. Data collected in all experiments were analyzed by SPSS version 17 and subjected to analysis of variance (ANOVA). The mean differences were tested using Duncan multiple range test (DMRT) with significant value of $P < 0.05$.

3. Results and Discussion

3.1. Asymbiotic Seed Germination

The disinfecting method was 100% successful in eliminating any seed contamination.

Germination tests are performed under well-defined conditions where all factors are controlled. The germination of *Cladanthus mixtus* was carried on distilled gelled water and Gautheret (1959) medium. Trace output is shown in the following Figure 1:

The two basal medium which has been tried with different nutritional composition, gelled distilled water After 33 hours, 100% of the seeds germinated, as considered by emission of the first radical in gelled water.

The maximum rate of germination (100%) is reached just after 34 hours in the medium containing the Gautheret (1959) medium.

The similarity of results can be explained by the ability of achenes *Cladanthus mixtus* to germinate in different environments.

Seeds of *C. mixtus* exposed to gelled distilled water medium showed higher germination percentage than for exposure to Gautheret (1959) medium.

This varied response in seed germination in different media might be due to the media compositions as both the media contains very low amount of macro and the micro elements and vitamins compared to Gautheret (1959) and gelled distilled water.

There is no significant effect of the composition of the medium on the germination of seeds *Cladanthus mixtus* L. So seeds germination does not require specific nutrient solutions to germinate, and Gautheret (1959) positively influenced seed germination and subsequent seedlings development (Figure 1).

Table 3. Macroelements composition (mg/l and mM) of different media used during cultivation of shoot of *Cladanthus mixtus* (L.)

Macroelement	MS (1962)		B5 (1968)		SH (1972)		SD (1980)		MSm (1982)	
	mg/l	mM	mg/l	mM	mg/l	mM	mg/l	mM	mg/l	mM
NH ₄ NO ₃	1650	20.6	-	-	-	-	500	6.25	275	3.4
CaCl ₂ ·2H ₂ O	440	3	150	1.02	200	1.62	530	3.6	220	1.5
Ca(NO ₃) ₂ ·4H ₂ O	-	-	-	-	-	-	-	-	-	-
KH ₂ PO ₄	170	1.25	-	-	-	-	100	0.73	85	0.62
MgSO ₄ ·7H ₂ O	370	1.5	500	2.04	399.24	1.64	614	2.49	185	0.75
KNO ₃	1900	18.8	3000	29.76	2500	24.73	1000	9.89	950	9.4
KCl	-	-	-	-	-	-	-	-	-	-
(NH ₄)H ₂ PO ₄	-	-	-	-	300	2.61	-	-	-	-
(NH ₄) ₂ SO ₄	-	-	134	1.01	-	-	-	-	-	-
NaH ₂ PO ₄ ·H ₂ O	-	-	150	1.24	-	-	-	-	-	-
Na ₂ SO ₄	-	-	-	-	-	-	-	-	-	-

Table 4. Ionic composition (mEq/l) of five macro-solutions tested.

Media	MS (1962)	B5 (1968)	SH (1972)	SD (1980)	Badoc (1982)
Cations					
Mg ²⁺	3.0	2.0	3.2	5.0	1.5
Ca ²⁺	6.0	2.0	2.7	7.2	3.0
NH ₄ ⁺	20.6	2.0	2.6	6.2	3.4
K ⁺	20.0	24.7	24.7	10.6	10.0
Na ⁺	-	1.1	-	-	-
Anions					
SO ₄ ²⁻	3.0	4.1	3.2	5.0	1.5
Cl ⁻	6.0	2.0	2.7	7.2	3.0
NO ₃ ⁻	39.4	24.7	24.7	16.1	12.8
H ₂ PO ₄ ⁻	1.2	1.1	2.6	0.7	0.6
Total Azote	60.0	27.8	27.6	22.4	16.25
Total Ions (mEq/l)	99.3	63.8	66.6	58.1	35.8

Table 5. Effects of five standard macrosalt formulations on shoot growth, rooting, and percentage of hyperhydrie and callogenesis of the wild chamomile Morocco: *Cladanthus mixtus* L. after 30 days. Numbers of shoots, root as well as shoot height and root length, percentage of hyperhydrie and callogenesis.

Basal media	Mean ± S.D.				% Hyperhydrie	% Callogenesis
	Numbers of shoots per explant	Shoot height (cm)	Number of root	Root length (cm)		
MS	2.60 ± 0.29 a	2.75 ± 0.12 a	3.33 ± 0.17 a	2.42 ± 0.16 a	0	0
SD	1.53 ± 0.18 b	2.12 ± 0.07 b	2.20 ± 0.36 b	1.15 ± 0.17 b	3.33	30
B ₅	1.70 ± 0.15 b	1.67 ± 0.04 c	1.97 ± 0.30 b	0.86 ± 0.21 b	16.66	100
MSm	1.37 ± 0.09 b	1.85 ± 0.08 c	1.70 ± 0.31 b	0.26 ± 0.06 c	33.33	100
SH	1.20 ± 0.07 b	2.10 ± 0.07 b	2.60 ± 0.18 b	0.93 ± 0.10 b	0	33

Means followed by the same letter along rows are not significantly different by Duncan's test ($P \leq 0.05$). MS: Murashige and Skoog (1962); SD: Shah and Dalal (1980); B₅: Gamborg and Eveleigh (1968); MSm: Murashige and Skoog modified (Badoc, 1982); SH: Schenk and Hildebran (1972).

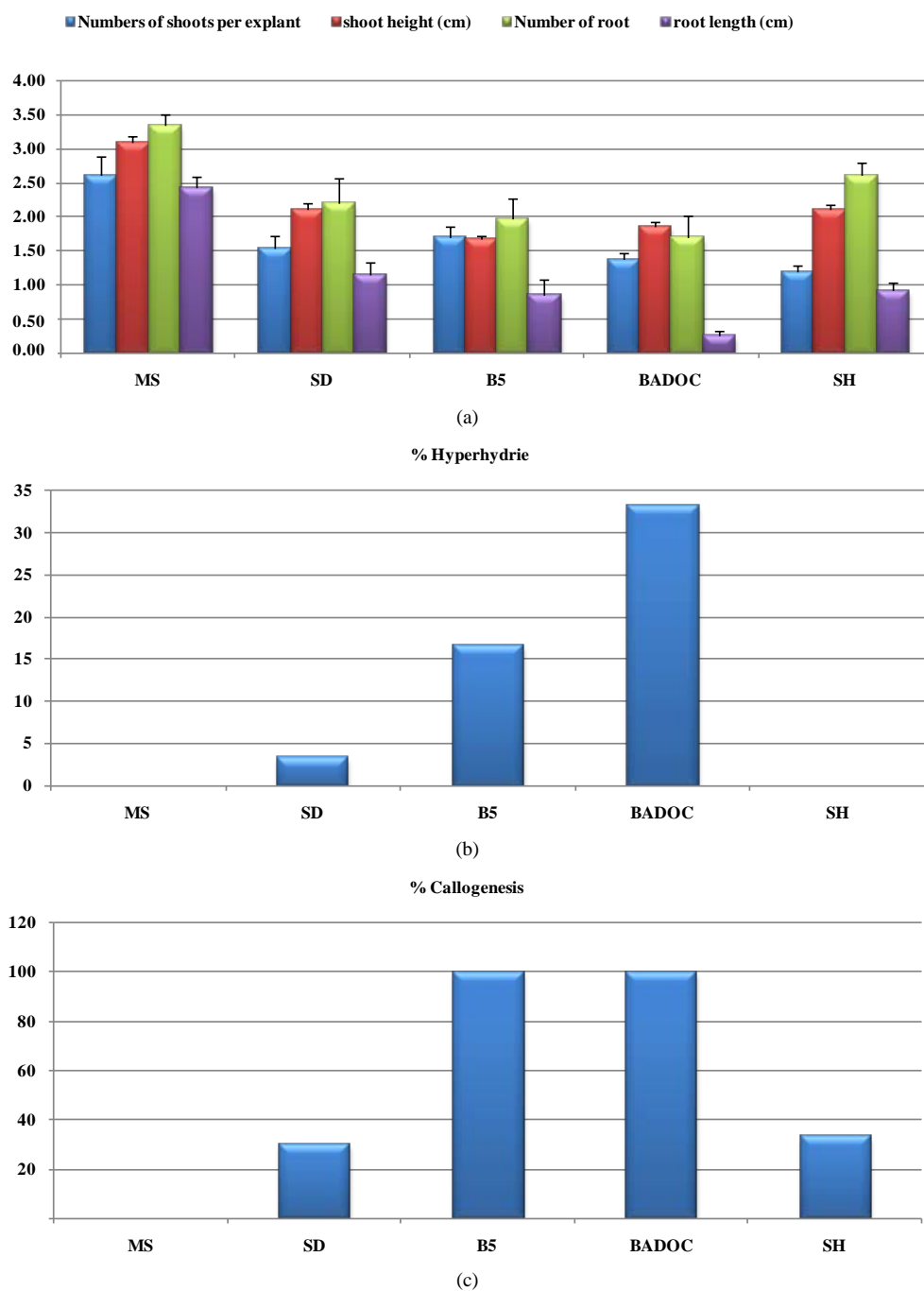


Figure 2. Influence of five macroelements on development, growth and rooting (a), the hyperhydrie (b) and the callus formation (c) of shoot (n = 30) collected explants of long clone (A) *cladanthus mixtus* L., after 4 weeks of culture.

3.2. Effet of Culture Media on Plantlet Development from Shoot Tips

We tested the effect of the mineral solution on explants of Morocco wild chamomile; five standard macrosalt formulations MS (1962), SD (1978), SH (1972), Badoc (1982) and B₅ (1968) were tested for their effect on shoot multiplication; the results are shown in [Table 5](#), [Figure 2](#) and [Figure 3](#).

Statistically significant differences ($P \leq 0.05$) were observed in shoot multiplication from explants cultured in five multiplication media containing different macroelements ([Table 5](#)).

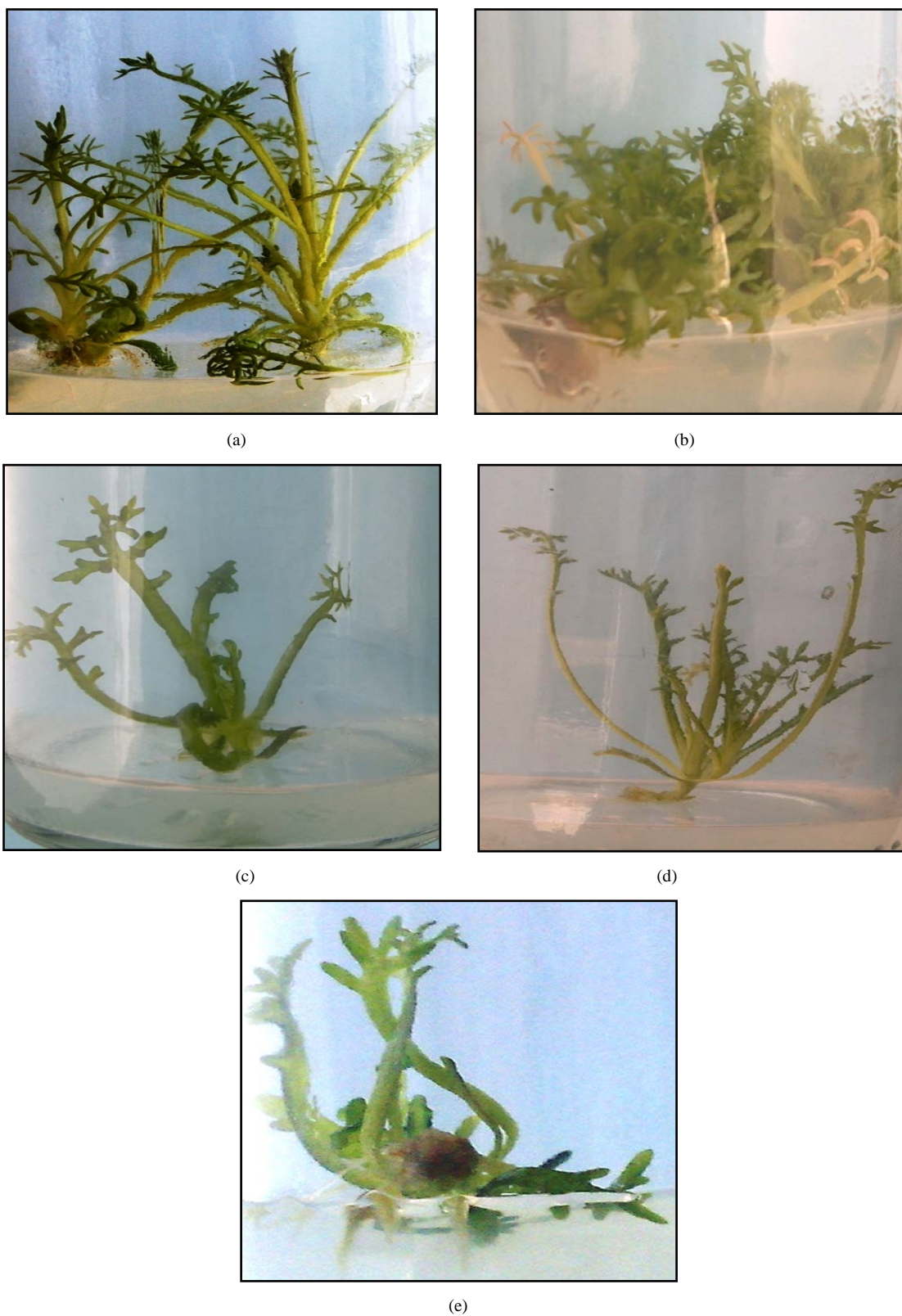


Figure 3. Plantlet of long clone (a) *cladanthus mixtus* L. developed on five macroelements media observed after 4 weeks of culture, (a) MS medium; (b) SH medium; (c) B₅ medium; (d) SD medium; (e) BADC medium.

Significant differences on shoot number between media were obtained in five multiplication media, with the highest multiplication rate obtained on MS medium. However, shoot number did not significantly increase in other media, as observed in MS medium (Figure 2(a)).

On the MS medium, the mean number of shoots per explants achieved 2.60 with a length of 2.75 cm, the mean number of roots achieved 3.33 per explants with a length of 2.42 cm, and the percentage of hyperhydrie and callus is absent (Figure 2(b), Figure 3(a)).

However, a significant reduction on the mean numbers of shoots, roots as well as shoot height and root length, was observed in other media as compared to MS medium.

Among the five solutions macroelements tested (Table 5), those of B₅ and MSm prove harmful to the explants; they induce a higher percentage of callus (100%) (Figure 2(c)) and the percentage of hyperhydrie is 16.33% and 33.33% respectively (Figure 3(c)).

MS, B₅ and MSm media do not induce callus against in the medium SD representing 30% of callus which will reach 100% in the SH medium (Figure 3(b)).

MS and SH media do not induce hyperhydric plants against by the SD medium which causes a low percentage of hyperhydrie (3.33%), which increases to 16.33% on B₅ and 33.33% on MSm medium.

So according to our results macronutrients MS (1962) is more favorable to the multiplication, growth and rooting of shoots of *Cladanthus mixtus* L. and is used for the following tested growth regulators.

The success of *in vitro* culture of plant species is influenced by several factors including the culture medium [25] [26]; we can say that it is the concentration of nitrate that gave the difference between the five culture media. The decrease of N and K in other media and especially MSm (Badoc) results in a low shoot elongation of clone Studied.

The difference between the number and length of the shoots and roots of the clone A in five culture media can be explained by the difference between the concentrations of these minerals elements.

This difference is explained by [27] which showed that the MS (1962) medium is mainly characterized by very high nitrogen content that the third party is brought in reduced form (NH_4^+); this chemical element is the fundamental component of the living matter.

The presence of a high concentration of potassium (K^+) in the MS medium plays a role in the induction of organogenesis, especially for the neoformation of buds, stem elongation and the production of biological materials.

These same results were observed with [28]-[36], who have successfully observed a micropropagation of *Matricaria recutita* and *d'Anthemis nobilis* L. on Murashige and Skoog (1962) medium, with a very high percentage of shoots.

Although more than 50 different devised media formulations have been used for the *in vitro* culture of tissues of various plant species [37] [38], the formulation described by Murashige and Skoog (1962) (MS medium) is most commonly used, often with relatively minor changes ([39]-[46]).

This explains the ability of this species to grow better in the MS (1962) medium as another medium.

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