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Effects of phytohormones on seed production in *Ananas comosus* Merril var. *Cayenne* by *in vitro* culture techniques

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ABSTRACT

Ananas comosus (Bromeliaceae) is a purely tropical perennial herb whose fruits are appreciated. The cultivated species is a self-incompatible diploid and produces few seeds. The aim of the present study is to investigate the in vitro culture conditions favorable to the rapid and mass regeneration of Ananas comosus plants in order to establish a protocol for propagation. The explant used is the apices from the crown. After disinfection, the apices taken from the crowns of Ananas comosus var. cayenne are cultured on a complete Murashige and Skoog (MS) medium containing different concentrations of Benzylaminopurine (BA), Kinetin (KIN), 2,4-Dichlorophenoxyacetic acid (2,4-D), Naphtaelene acid (NA), as well as combinations of BA/NAA and BA/2,4-D were studied. NAA promotes callogenesis with maxima of 87.5% with 3 or 4 mg.l^{-1} versus 100% in the presence of 1.5 or 2 mg.l^{-1} 2,4-D. BA at 2 mg.l^{-1} and KIN at 3 mg.l^{-1} allowed the proliferation of more than 100 buds per cal. The BA/NAA ratio at 2/2 mg.l^{-1} allowed rooting and neoformation of leaves of vitroplants with a mean maximum number of 11.16 ± 1.4 leaves and 8.8 ± 1.2 roots per vitroplant and 11.41 ± 2.00 leaves and 9.8 ± 2.5 roots in the presence of 2/3 mg.l^{-1} BA/2,4-D. Some seedlings were acclimatized on a vermiculite soil mixture. Acclimatization of the vitroplants for 70 days was 86% successful. Apices are good starting materials for the indirect regeneration of Ananas comosus. Their use allows the regeneration of a large number of vitroplants and thus increases the potential for the in vitro production of healthy plant material in A. comosus.

Keywords: *Phytohormones, Ananas comosus, in vitro culture, vitroplants.*

Introduction

The genus Pineapple, of the family Bromeliaceae, has several species. However, all the varieties or cultivars currently exploited for their fruit for fresh consumption and for the food or pharmaceutical industries belong to only one of them: *A. comosus* [1]. In Cameroon, the production of pineapple for export has increased by 97% in recent decades.

Pineapple, *Ananas comosus* L. Merril is a perennial plant, native to tropical and subtropical America. The genus Ananas includes several species. However, all the varieties and cultivars currently exploited for their fruits for fresh consumption and for the food and pharmaceutical industries belong to only one of them: *Ananas comosus* [1]. The cultivated species is a self-incompatible diploid and produces few seeds. Commercial production is mainly based on six (6) var. Smooth Cayenne has become the mainstay of the pineapple industry because of its yield potential and its qualities, both for fresh and canned.

The export-import facility has enabled the market to increase to 10 million tons per year [2].

Pineapple production is limited by the scarcity of seeds and by the attack of parasites, particularly fungi of the genus Phytophtora and fruiting bodies. The lack of plant material of good sanitary quality is one of the major constraints to the extension and sustainability of pineapple plantations in the producing countries [3]. *In vitro* culture is a multiplication technique that makes it possible to quickly have planting material of good sanitary quality. These qualities are sought in the context of the rapid multiplication of new selected varieties and the renewal of plantations. It remains a possible way for the improvement of this species. The main objective of this work is to research the conditions of in vitro culture favorable to the rapid regeneration of *Ananas comosus* plants in order to establish a protocol for propagation.

Materials and Methods

The biological material used consists of traditional cuttings of *Ananas comosus* var. *Cayenne* purchased in the markets of Yaoundé (Cameroon), which have been stripped of their crowns and old leaves. This material was washed under a stream of tap water for 10 minutes followed by a wash under a stream of distilled water for 2 hours then sanitized by soaking in Tween 20 to 1% for 5 minutes and in sodium hypochlorite 30% for 45 minutes finally three successive rinses of 5, 10 and 15 minutes respectively with sterile distilled water were made under a horizontal laminar flow hood (MECAPLEX) near the flame of a Bunsen burner.

The basic medium most indicated for the regeneration and growth of Ananas comosus explants is the complete Murashige and Skoog medium to which 30 g/l sucrose, 7 g/l agar and

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the vitamin complex of [4] were added. The pH of the medium is adjusted to 5.6 (pH meter METROHM) using a 0.1N HCl or 1N NaOH solution. The culture medium is dispensed into culture tubes (SCHOTT, 25 x 115 mm) at a rate of approx. 10 ml per tube and sterilized by autoclaving at 121°C for 20 minutes. Under a pressure of 1.6 kg x cm^{-2}. Seeding is done under a horizontal laminar flow hood (MECAPLEX) near the flame of a Bunsen burner. Before culture, the basal part of each explant, whose cells are killed by the aseptic solutions, is removed with a sterile scalpel. The cultures are stored in a culture chamber where the following conditions prevail: - temperature: 26±1°C;

- illumination fluorescence light, (OSRAM) 80mol $m^{-2}.s^{-1}$ and photoperiod: 16/8.

Experiments performed on cytokinin-enriched BM showed that NAA and 2,4-D promote callogenesis in A. comosus. Thus, ANA added to complete BM at 1; 1.5; 2; 2.5 and 3 $mg.l^{-1}$ and complete BM at 0.5; 1; 1.5; 2; 2.5 and 3 $mg.l^{-1}$ made it possible to study the influence of these phytohormones on callogenesis.

For all these trials, the phytohormone-free medium is considered a control. Twenty-four healthy explants are cultured in normal polarity for each concentration of NAA or 2,4-D, and the assay is repeated three times. At the end of each experiment, which lasts approximately 35 days, the percentage of callogenes is recorded for each concentration of NAA or BA.

The results are processed by analysis of variance, and the significantly different means are separated by the Student test at the 5% probability threshold. Calluses obtained from pineapple apices are transplanted separately onto the budding media, which are made up of:

- Complete MS + Morel and Wetmore vitamin + 30 $g.l^{-1}$ sucrose + 7 $g.l^{-1}$ agar (MB) to which 0; 0.5; 1; 1.5; 2 and 3 $mg.l^{-1}$ BA have been added MB with the addition of 0; 0.5; 1; 1.5; 2 and 3 $mg.l^{-1}$, pH 5.6.

Twenty-four calluses are cultured for each concentration of BA or KIN, and the experiment is repeated twice. At the end of the culture (60 days), the percentages of budding calluses are recorded for each concentration of BA or KIN.

Similarly, the mean number of buds newly formed by callus is noted in each medium.

The buds induced on insulated wedges are separated under the horizontal laminar flow hood near the flame of the Bunsen burner. They are then transplanted into culture tubes containing the regeneration media. The regeneration media consist of a base medium enriched with different concentrations of BA/NAA or BA/ 2,4-D.

The pH of all media is adjusted to 5.6. All cultures are placed under the same conditions as described in paragraph 3. For each ratio of BA/NAA or BA/2,4-D, twenty-four buds formed are transplanted. Observations are made every 10 days for 120 days. On this date, the following growth parameters are recorded in each case: the number of newly formed leaves per seedling and the number of roots formed per seedling.

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- BM with the addition of 0; 0.5; 1; 1.5; 2 and 3 mg.l-1, pH 5.6.

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The 2-month-old vitroplants are transplanted in 18 cm high and 12 cm diameter perforated polyethylene bags filled with a

After 20 days, the transparent lids are removed, and the pots are placed outside under a shelter that protects the seedlings from too much rain and sunshine. The seedlings are maintained in these conditions for 15 to 20 days.

The seedlings are removed from the shelter and placed in the open air. After 30 days, they are ready to be transferred to the field. At the end of each stage, the dead plants are counted in order to evaluate the survival rate.

Results

Indirect budding

Callogenesis

In *A. comosus*, callogenesis is induced from the apices on MS medium. After 30 days, friable and yellowish calluses are obtained in the presence of NAA and 2,4-D (Fig.2). The percentage of callogenesis varies according to the concentration of different phytohormones in the medium. The percentage of callogenesis increases from 8.33% in the presence of 0.5 $mg.l^{-1}$ NAA to a maximum of 87.5% with 3 or 4 mg.l-1 (Fig. 2A). Above this concentration, the percentage of callogenesis decreases to a minimum of 37.5 $mg.l^{-1}$ with 5 $mg.l^{-1}$ NAA (Fig. 1A).

With 2,4-D, the callogenic percentage increases from 20% with 0.5 $mg.l^{-1}$ to a maximum of 100% with 1.5 or 2 $mg.l^{-1}$ (Fig. 2B). Above this concentration, the callogenic percentage decreases to a minimum of 41.6% with 3 $mg.l^{-1}$ (Fig.1B).

These calluses maintained in the same medium for 60 days differentiate a few buds on average 10 ± 2 that can be transplanted to a regeneration medium after isolation. In the absence of NAA and 2,4-D (0 $mg.l^{-1}$), no callus is formed (0%). These auxins are essential for organogenesis.

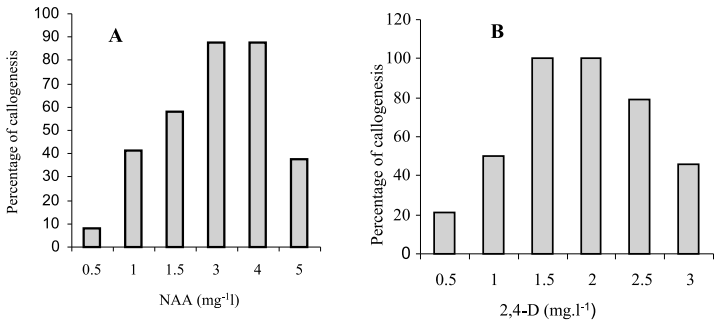

Fig. 1: Effects of NAA (A) and 2,4-D (B) on callus induction from apices calluses isolated from A. comosus crowns after 30 days of culture.

Effect of BA on the proliferation of buds on calluses

In *A. comosus*, apices-derived calluses are grown for 30 days on complete MS medium with the addition of 0 to 5 mg.l^{-1} BA. The percentage of budding callus increases from 40.27 ± 4.80% with 1 mg.l^{-1} BA to a maximum of 100 ± 2% with 2 mg.l^{-1}. Above this concentration, the percentage decreases to a minimum of 63.88 ± 20.97 mg.l^{-1} with 5 mg.l^{-1} BA.

The mean number of buds formed per cal in the presence of BA increases from 29.33 ± 3.05 in the presence of 1 mg.l^{-1} to a maximum of more than 100 buds with 2 mg.l^{-1} (Fig. 2C, and D). Above this concentration, the number decreases to a minimum value of 46.33 ± 3.15 mg.l^{-1}. (Table 1). These buds maintained in the same medium for 60 days develop into seedlings and take on a dark green appearance (Fig. 2E).

Table 1. Effect of BA concentration on proliferation and mean number of buds per callus in A. comosus after 30 days of culture

Concentration of BA (mg.l^{-1})	Percentage of calluses badding	Average number of buds formed by cals
0	0	0
1	40.27 ± 4.80^{d}	29.33 ± 3.05^{d}
2	100.00 ± 0.00^{a}	> 100^{a}
3	80.55 ± 8.67^{c}	64.00 ± 4.00^{b}
4	65.27 ± 4.80^{b}	58.33 ± 3.51^{b}
5	63.88 ± 20.97^{b}	46.33 ± 3.15^{c}

Values with the same letter in the same column are not significantly different according to Student's test (p<0.05).

Effect of kinetin on the proliferation of buds on calluses

With this phytohormone, the percentage of budding increases from 54.16 ± 4.16% in the presence of 1 mg.l^{-1} to a maximum of 86.10 ± 8.67% with 3 mg.l^{-1} KIN (Fig. 2F). Above this concentration, the percentage decreases to a minimum value of 51.38 ± 8.67% with 5 mg.l^{-1} (Table 2). The average number of buds formed increases from 40.33 ± 7.63 with 1 mg.l^{-1} of KIN to more than 100 buds with 3 mg.l^{-1}. Above this concentration, this number decreases and reaches a minimum value of 52.00 ± 11.13 buds per cal with 5 mg. Newly formed buds are obtained after 30 days of culture (Fig. 2F.). These buds maintained in the same medium for 60 days develop into root-bearing seedlings (Fig. 2G.)

Table 2. Effect of kinetin concentration on bud proliferation and mean number of buds per callus in A. comosus after 30 days of culture

Concentration of KIN (mg.l^{-1})	Percentage of calluses badding	Average number of buds formed by cals
0	0	0
1	54.16 ± 4.16^{d}	40.33±7.63^{bc}
2	77.77 ± 6.36^{b}	57.00±9.83^{b}
3	86.10 ± 8.67^{a}	> 100^{a}
4	63.88 ± 4.80^{c}	66.66 ± 7.57^{bc}
5	51.38 ± 8.67^{d}	52.00 ± 11.13^{b}

Values with the same letter in the same column are not significantly different according to Student's test (p<0.05)

Figure 2: Morphological aspects of callus induced from the apices (A: MB + 4 mg.l^{-1} NAA; B: MB + 2 mg.l^{-1} 2,4-D (dash =1cm) after 30 days of culture and effects of BA (A, B and C: Budding induced on MS + 1.5 and 2 mg.l^{-1} BA respectively) and effect of kinetin (D and E: Buds induced on MS + 2 and 3 mg.l-1 BA and KIN) on bud proliferation from callus after 30 days of culture.

Regeneration of Pineapple comosus from new buds on callus.

The buds formed on pineapple callus are isolated and transplanted under the same conditions as on the regeneration media in order to transform them into complete vitroplants.

Effects of BA/NAA combinations

A. comosus buds are isolated from the callus and transplanted onto complete MS medium containing 2 mg.l^{-1} BA and 1 to 5 mg.l^{-1} NAA. All transplanted buds resume growth within 10 days. After 60 days of culture, the best development is obtained with the 2/2 ratio (2 mg.l^{-1} BA and 2 mg.l^{-1} NAA). The vitroplants under these conditions have an average of 11.16 ± 1.4 newly formed leaves and 8.8 ± 1.2 roots (Table 3, Fig. 3A). Other hormonal ratios give lower results.

Table 3. Effects of NAA concentration on the regeneration of A. comosus from cals neoformed buds after 60 days of culture on BM in addition to 2 mg.l^{-1} BA

Concentration of BA/NAA (mg.l^{-1})	Percentage of calluses badding	Average number of buds formed by cals
2/1	10.50 ± 2.1^{c}	6.6 ± 2.3^{c}
2/2	11.16 ± 1.4^{a}	8.8 ± 1.2^{a}
2/3	7.33 ± 2.0^{c}	7.6 ± 1.9^{b}
2/4	10.00 ± 1.4^{b}	6.6 ± 1.9^{c}
2/5	7.66 ± 2.1^{c}	7.4 ± 1.6^{b}

Values with the same letter in the same column are not significantly different according to Student's test (p<0.05).

Effects of BA/2,4-D combinations

A BA/2,4-D ratio of 2/3 (2 mg.l^{-1} BA and 3 mg.l^{-1} 2,4-D) resulted in the best vegetative growth. After 60 days of culture, the vitroplants had an average of 11.41 ± 2.0 newly formed greenish leaves and 9.8 ± 2.5 roots (Table 4, Fig. 3A and B). All other ratios tested yielded relatively poor results.

Table 4. Effects of 2,4-D concentration on the regeneration of A. comosus from neoformed buds after 60 days of culture on MS in addition to 2 mg.l^{-1} BA

Concentration of BA/2,4-D (mg.l^{-1})	Number of neoformed sheets/vitroplant (mean ± standart deviation)	Number of neoformed roots/vitroplant (mean ± standart deviation)
2/1	8.50 ± 1.8^{c}	8.4 ± 2.6^{b}
2/2	11.00 ± 1.8^{a}	8.8 ± 1.9^{b}
2/3	11.4 ± 12.0^{a}	9.8 ± 2.5^{a}
2/4	10.16 ± 2.1^{b}	8.6 ± 2.5^{b}
2/5	9.32 ± 1.2^{c}	8.2 ± 3.9^{b}

Values with the same letter in the same column are not significantly different according to Student's test (p<0.05)

Regeneration rate and acclimatization of vitroplants

Using BA at 2 mg.l^{-1} on callus, on average more than 100 buds per callus are induced from the apices fragments of *A. comosus*. Using 3 mg.l^{-1} KIN, more than 100 buds per callus are induced on the same explants. When these buds are transplanted into the same enriched basal medium with a BA/NAA ratio of 2/2 and a BA/2,4-D ratio of 2/3, whole vitroplants are regenerated. Out of 50 vitroplants acclimatized for 70 days, 43 survive acclimatization, i.e. 86% success (Fig. 3C) and all survivors are transferred to fields (Fig. 3D).

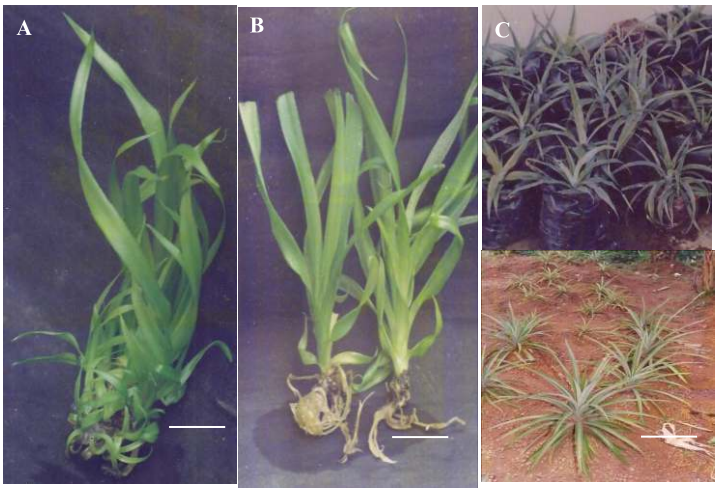

Figure 3. Pineapple seedlings regenerated after 60 days of cultivation (Figs. 3A and B) and acclimatized after transplanting on soil and vermiculite (v/v) substrate after 70 days (Fig. 3C) and transferred to the field (Fig. 3D) (dash = 1.5 cm).

Discussion

The classical method of pineapple propagation is limited due to the low number of shoots (0 to 3) produced per plant and in the field (Anonymous, cit. [1]. The *in vitro* culture techniques currently used for the rapid propagation of this plant allow the production of a large number of seedlings and their sanitation [5]. These techniques make it possible to circumvent the difficulties of conventional vegetative propagation [6]. The aim of this study was to study in vitro the potentialities of *A. comosus* seed production in order to establish a protocol for regeneration. The following steps were studied during this process: callogenesis, bud induction, regeneration from buds newly formed on callus, and acclimatization of regenerated plants. However, indirect micropropagation of pineapple was carried out using the apices of the crown. The micropropagation of species by induction of buds on callus is not a new technique.

Indeed, many authors had already advocated the use of the juvenile state of explants from various sources to induce callogenesis and bud neo-formation [7]; [8] and [9]. The auxins used to induce callogenesis on pineapple explants in this work are 2,4-D and NAA. This choice is also due to the fact that these two phytohormones, apart from their rhizogenicity, are used to induce callogenesis in most species [9]. According to these results, the percentages of callogenesis obtained vary considerably depending on the concentration of auxins in the culture medium. Thus, under optimal conditions, 100% of the apices cultured form callus in the presence of 1.5 or 2 mg.l^{-1} 2,4-D versus 87.5% in the presence of 3 or 4 mg.l^{-1} NAA. Similar results were obtained by [10] from pineapple apices. The presence of these phytohormones in the culture medium is essential for callogenesis to take place. This callogenesis effect has been demonstrated in Irvingia gabonensis and Irvingia wambulu [11]. Induction of the proliferation of burghers on the callus of the species studied was obtained with BA and KIN. Cytokinins in general, BA and KIN in particular, are recognized as phytohormones that promote organogenesis in general and bud formation in particular in in vitro culture of different species [12]. The percentages of budding callus and the average number of induced buds vary significantly with the concentration of BA and KIN in the culture medium [9]. Under optimal conditions, there are on average more than 100 buds per callus, and 100 ± 2% of calluses from the apices form buds in the presence of BA. Similar results were obtained by [13] with pineapple apices in the presence of AIA and BA. With KIN, 86.10 ± 8.67 % of calluses from the apices give buds. Each callus bears, under optimal conditions, more than 100 buds. These results are comparable to those obtained by [14]; [15], who conducted work on the in vitro regeneration of pineapple from apices grown in semi-liquid medium. Whole vitro plants are regenerated from the newly formed buds. This regeneration requires a simultaneous supply of BA and NAA or BA and 2,4-D. The simultaneous contribution of a cytokinin and an auxin in the regeneration of vitroplants has been demonstrated in several species [9]. The two types of phytohormones act in complementarity [16]. The cytokinins would, in general, support the development of the vegetative port while the auxins would support rhyzogenesis [17]. For these results, the BA/NAA ratio equal to 2/2 is more favourable to the formation of rooted vitroplants from proliferated buds on cal. With the BA/2,4-D equilibrium, it is the 2/3 ratio that is the most favourable to the formation of rooted vitroplants. Indeed, at the end of the cultures (120 days), all the transplanted buds formed rooted vitroplants with an average of 7.66 ± 2.1 to 11.41 ± 2.00 leaves and 6.6 ± 2.3 to 9.8 ± 2.5 roots. These results are similar to those obtained by [18] in carob (*Ceratonia siliqua*). The success rate is determined by the acclimatization conditions of the regenerated in vitro plants. In this work, a success rate of 86% of the vitroplants was obtained from buds from callus. This success rate is generally noted in a few species, such as *A. comosus* [1] and macabo [19].

Conclusion

Indirect *in vitro* micropropagation of *Ananas comosus* var. cayenne was carried out from the apices of the crown. The main objective of the work was to study *in vitro* the potentialities of *A. comosus* seed production in order to establish a protocol for propagation. In *in vitro* culture, the effectiveness and success of regeneration depend on several factors such as the choice of the explant, the type of basic medium, the choice of phytohormones, and the search for hormonal balance of the culture medium.

In this work, the production of *A. comosus* seeds *in vitro* was first performed by callogenesis, which is induced with NAA and 2,4-D and the percentage of success varies according to the concentration of NAA or 2,4-D in the culture medium, then by the neoformation of buds on cal. This step is determined by the different concentrations of BA and KIN, then the regeneration of the vitroplants from the neoformed buds. This phase requires a hormonal equilibrium between BA and NAA or between BA and 2,4-D. It specifically leads to the production of rooted vitroplants. Acclimatization, which is the final phase, depends on the environmental conditions and especially on the rooting of vitroplants. Under the favorable conditions defined in our work, after acclimatization of the vitroplants, the success rate is 86% after 70 days of culture.

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