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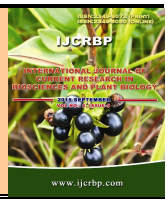

Original Research Article

Morphological and Physiological Characterization of Some *Vernonia* spp. Pollen Genotypes in Cameroon

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Abstract

Given the importance of non timber forest products like some species of *Vernonia* used for food and medicinal purposes, it is necessary to determine the morphological and physiological characteristics of their pollen grains needed for enhanced reproduction. Morphological studies have proven that pollen grains of *Vernonia* spp. are fenestrate, echinate, porate as well as spherical. The aim of this research was to address the diversity observed within the genus *Vernonia* based on additional proves. Conditions of pollen grain germination and its viability and conservation were thus analysed. Results show that the largest pollen grains were obtained from *V. calvoana* var. *microcephala* (69.42 µm long and 66.30 µm wide). *V. amygdalina* has the smallest size (49.30 µm in length and 44.30 µm in diameter). The study on pollen germination conditions show that *Vernonia spp.* grows exclusively on solid medium. Improved Brewbaker and Kwack medium was the main germination medium for *V. amygdalina* pollen. On the contrary, an improved Heslop-Harrison medium was appropriate for the germination of *V. calvoana* pollen. It was observed that pollen of the genus *Vernonia* mature before the opening of the stigma and survives for only three hours.

Keywords

Conservation

Culture medium

Morphology

Pollen germination

Vernonia spp.

Introduction

Asteraceae is one of the four largest and diversified families of plants with 1535 genera and 23000 species (Ehab, 2001). In Cameroon, the genus *Vernonia* is one of the representative genera of the *Asteraceae* family with 19% of the species (Biholong, 1986). Some species like *V. amygdalina* Del. and *V. calvoana* Hook classified as non timber forest products are used for food and medicinal purposes. Extracts of *V. colorata* have shown

hypoglycaemic and antidiabetic properties (Sy et al., 2008). Leaf extracts of *V. amygdalina* have been used in various traditional medicines against helminthic, protozoal as well as bacterial infections with scientific evidence of these claims (Farombi and Owoeye, 2011). Phytochemicals such as saponins and alkaloids, terpens, steroids, coumarins, flavonoids, phenol acids, lignans, xanthones, anthraquinones, edotides and sesquiterpenes have been isolated from *V. amygdalina* (Farombi and Owoeye, 2011). These compounds elicit various biological effects including cancer chemoprevention effect. The increase in population continues to raise the number of consumers leading to an over exploitation of *Vernonia spp*. There is therefore great need to carry out conservation studies in order to protect these important genetic resources from extinction.

From a taxonomic point of view, the *vernonieae* are considered as one of the most complex groups within the *Asteraceae*. The species of this group vary greatly in habit and morphology thus different criteria for taxonomic delimitation at the generic and infra-generic levels have been adopted (Angulo and Dematteis, 2009). In addition, a few biochemical, palynological, and genetic studies have been carried out on these plants (Donfack et al., 2007), some revealing many perceptible variations in pollen morphology within the genus (Dematteis and Salgado, 2001).

Studies on nutritional qualities of *V. amygdalina* have been carried out but very little work is known on its agronomic practices or genetic enhancement (Apabode and Adebooye, 2005). In order to improve knowledge on their identification and reproduction, it is important to develop and promote the physiological and morphological investigations of these plants. Moreover knowledge on their reproduction ability can be used to establish a plan for the selection and genetic improvement of *Vernonia*. In this light, studies on pollen germination are very useful especially to control reproduction of members of the genus *Vernonia*. *Vernonia amygdalina* is one of the species chosen in this study due to its ubiquitous characteristics that make it adapt to diverse climatic conditions. Its leaves are always much more bitter than those of *V. calvoana* for example. Three taxa of *V. calvoana* were retained for the study. One has only white florets, another purple and the third small purple florets. They grow in mountain areas in humid equatorial conditions. This study aims to establish preliminary data with new insights on pollination occurrence in *Vernonia* species in order to provide evidence on diversification within the genus. Conditions of pollen grains germination and viability are also investigated.

Materials and methods

Materials

Pollen grains of two species of *Vernonia* were examined namely: *V. amygdalina* and *V. calvoana*. Among *V. calvoana* species three taxa were of interest: *Vernonia calvoana* (Hook. f.) Hook. f. var. *calvoana* presenting a big capitulum with purple florets and *V. calvoana* (Hook. f.) Hook. f. var. *calvoana* presenting big capitulum with white florets. The third taxon is *V. calvoana* var. *microcephala* C.D. Adams with a small capitulum presenting purple florets.

Methods

Morphological traits of pollen

This study was carried out from October, 2009 to January, 2010. Flowers were produced during this period. Three months from October, seeds of *V. calvoana* were sown and cultivated in an experimental farm in Yaounde.

Pollen was isolated from anthers derived from various florets of different fresh capitula of the same plant taxon. Pollen grains from each of the different taxa were acetolysed using Erdtman (1952) method. After acetolysis, pollen was observed under an optical microscopy to describe it and to measure equatorial and polar diameters (Fig. 1).

Harvesting and *in vitro* culture of pollen

Pollen harvest

Capitula were cut from the farm at 6:00 AM. The species were isolated from each other and taken to the laboratory. The florets were then removed from the capitulum and the anthers extracted. As precaution, pollen used was left on the anthers to prevent infection and to ensure taxon identity. The anther with the pollen was immediately put in a culture medium deposited on a glass slide. The latter was placed in a Petri dish under limited humidity, and incubated at 20°C. Three replicates were used for each treatment and each replicate contained at least 500 but not more than 800 pollen grains per slide.

Pollen germination media

Two types of germination media were tested: liquid medium and solid medium by adding 1% agar. Both were prepared with a Brewbaker Kwack (BK) (1963) solution for the first experiment and with Heslop-Harrison (HH) (1977) solution in the second. In a preliminary investigation, media having BK salts at different concentrations [from 0 to 50% of sucrose in first liquid medium and 0, 5, 10,…, 50% in the second solid medium] were screened. The experiment was repeated with HH solution. All the four *Vernonia* genotypes studied were involved in this preliminary step which helped to select a basal germination medium for each of the varieties. The incubation took 24 h at 20°C. The basal medium selected for one variety was one with the best percentage of pollen germination. A pollen grain was considered germinated if its pollen tube was longer than wide (Jayaprakash and Sarla, 2001). After incubation, cultures were fixed and colored, for better observations under an optical microscope by Allexander (1969) solution. This basal medium was then used in the subsequent tests but further improved by altering the temperature, pH, boric acid concentration, and developmental stage of the floret.

To investigate the viability of pollen, the pollen life span in different storage conditions of 25°C, 10°C (inside refrigerator) and 0°C was observed and, two principal developmental stages of the floret corresponding to two different developmental periods of pollen grains were distinguished. In stage I, the capitulum was matured but with closed petals and anthers. In stage II, capitulum was matured; petals were also opened with an anther by a plunger pollination mechanism. Experimental pollen varieties were collected in those two conditions and cultivated on improved germination medium. In this study the evolution of pollen tube growth was also analyzed. Using an ocular micrometer, the lengths of 30 pollen tubes were measured to calculate mean pollen tube length after every additional hour of incubation beginning from the 22nd to the 29th hour of incubation.

Results

Pollen morphology

Microscopic observation shows that *Vernonia* pollen grains studied are spherical, porate, fenestrate and echinate (Fig. 1). The biggest pollen is that of *V. microcephala* while *V. amygdalina* has the smallest pollen grains (Table 1). Statistical analyses using Fisher test helped to establish significant differences between the sizes of pollen grains studied. It is also noticed that pollen of all those genotypes when shed out of anthers can be recovered by an adhesive and gelly white substance.

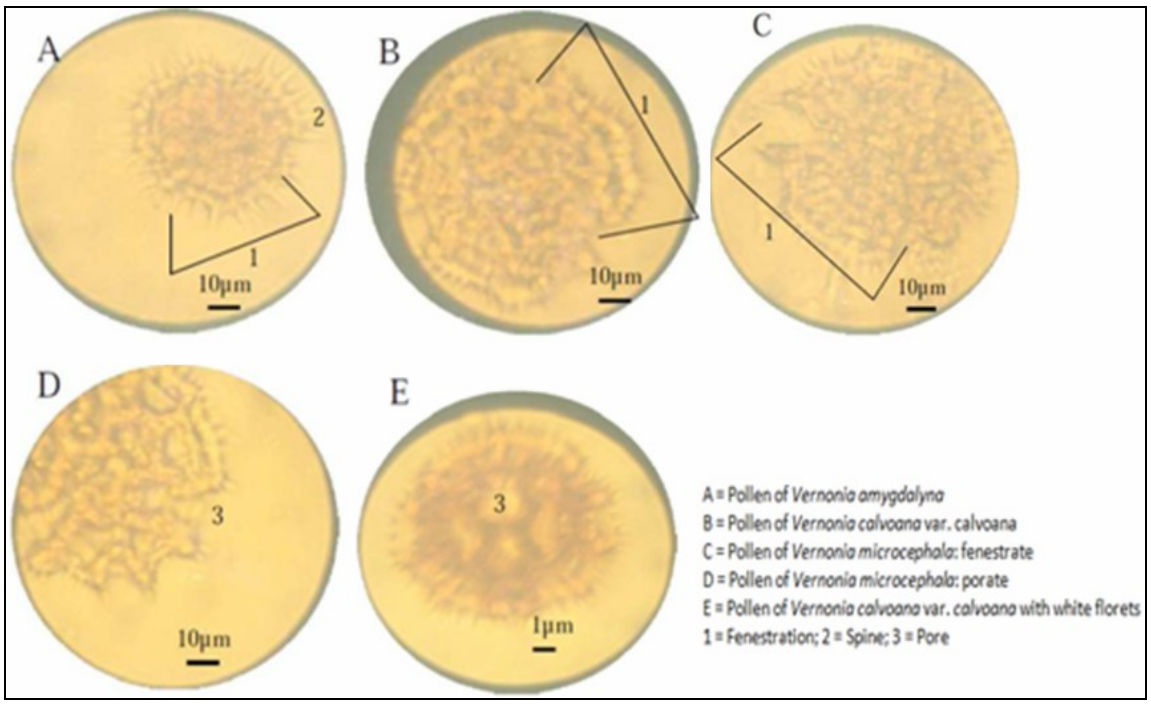

Fig. 1: Pollen of *Vernonia spp*. observed with an optical microscope.

Table 1. Summary of morphological characteristics of *Vernonia* pollen.

Species	E (µm)	P (µm)	P/E
V. amygdalina	44.30 ± 4.86	49.30 ±5.12	1.11
V. calvoana with white florets	62.40 ± 2.73	64.53 ± 2.82	1.03
V. calvoana var. *calvona*	65.42 ± 4.68	69.24 ± 5.36	1.05
V. microcephala	66.30 ± 4.42	69.42 ± 4.65	1.05
Equatorial length (E); Polar length (P); All pollen grains were spherical with porate apertures. The tectum structure was echinulate and fenestrate			

Determination of best growth medium and influence of sucrose concentration on pollen germination

Pollen grains of various genotypes studied here did not germinate in liquid medium in the presence or absence of sucrose. Germination test was successful only in solid medium and *Vernonia* pollen germinated in high concentration of sucrose. The optimum germination percentage was obtained with 25% sucrose for *V. calvoana* var. *calvoana*, *V. calvoana* var. *microcephala*, *V. amygdalina* (Fig. 2). The pollen of *V. calvoana* var. *calvoana* with white florets shows the best germination percentage at 35% sucrose concentration (Fig. 2c).

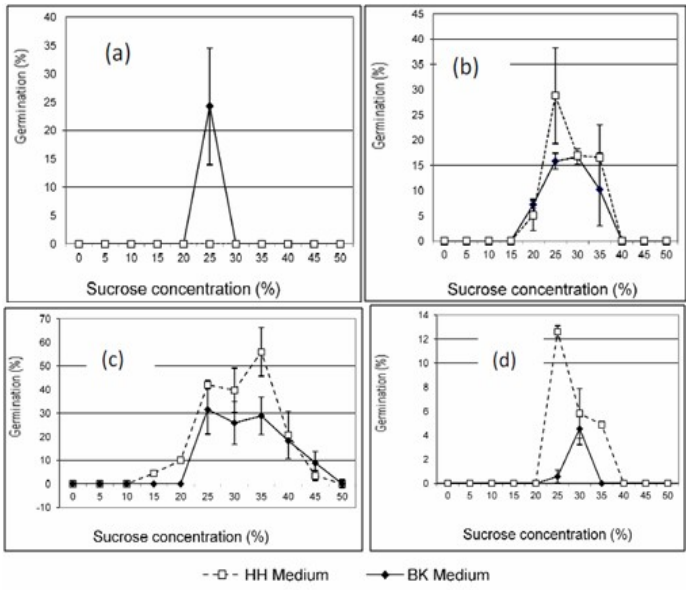

Fig. 2: Effect of sucrose concentration (W/V) on *in vitro* pollen germination of *Vernonia spp*. tested in two different basic media (HH and BK). (a) *V. amygdalina*; (b) *V. calvoana* var. *calvoana*; (c) *V. calvoana* var. *calvoana* with white florets; (d) *V. microcephala*

The male gametophyte of *V. amygdalina* is the only one that can germinate in BK medium due to its specificity. Moreover in the four genotypes studied, it is the only species with a difference.

The influence of temperature on pollen germination

V. calvoana var. *microcephala* produces pollen that is capable of germinating at 35°C. The three other genotypes retain their maximum germination capacity at 25°C (Fig. 3). In the ecological area where *Vernonia* spp. grows, temperatures of up to 25°C are not very common at night.

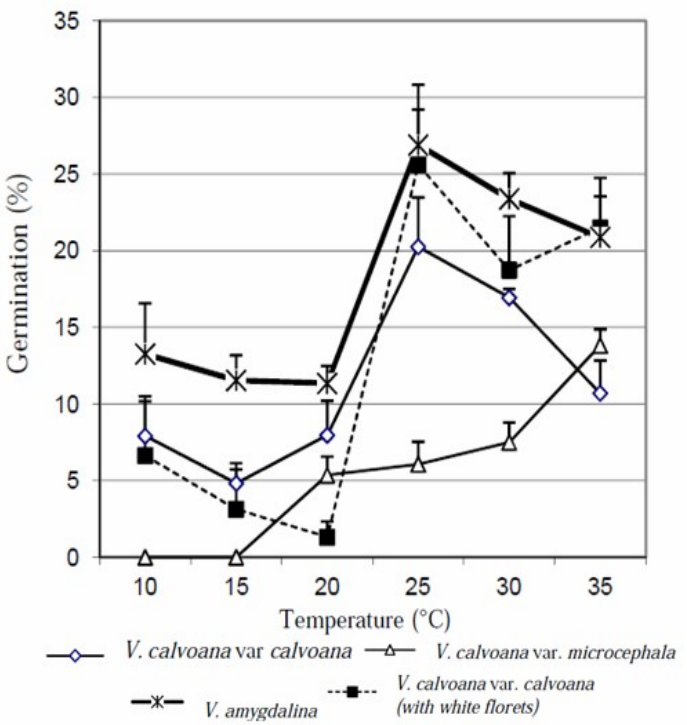

Fig. 3: The influence of temperature on pollen germination of *Vernonia spp*.

Determination of the appropriate developmental stage for collecting pollen from flowers

In the present study, stage I is the best stage to collect viable pollen from anthers (Fig. 4). At this stage, the pollen is still protected by the anther wall and petals from external stress. Thereafter, petals open and the pollen are pushed out of the anther by a plunger mechanism.

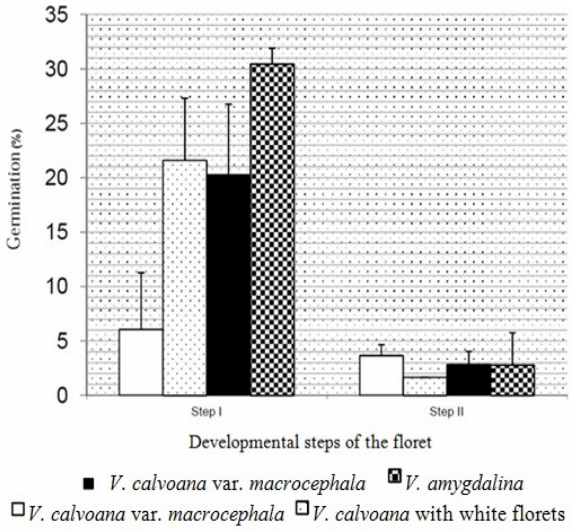

Fig. 4: Percentage germination of pollen grains of four *Vernonia* spp. genotypes collected at two developmental steps of the floret and cultivated on the best culture medium.

Influence of storage duration of pollen on germination

The percentage germination of pollen decreases with the duration of storage at room conditions (Fig. 5). Nine hours after collecting pollen, germination in artificial media is unsuccessful. *Vernonia* pollen has a very short life span. To control cross-pollination, this pollen is better used within less than an hour after its isolation from the plant to ensure an optimum viability of the male gametophyte. *Vernonia* pollen lives for 6 to 9 h when it is conserved with its capitulum under room conditions. When pollen is isolated from the anther, it loses its germination capacity three hours later.

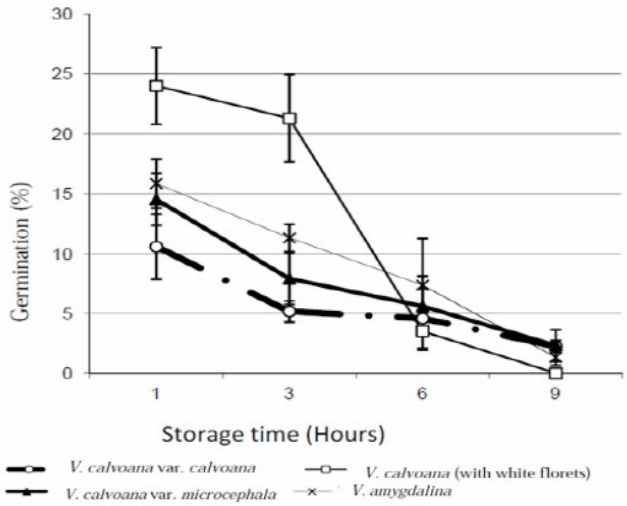

Fig. 5: Evolution of *Vernonia* pollen germination capacity with time spent at room temperature after harvest.

Effect of boric acid concentration on germination

Various concentrations of Boric acid included in the germination medium of *Vernonia* influence pollen germination capacity (Fig. 6). An addition of 0.8g/L of boric acid to the medium of *V. calvoana* var. *microcephala* produced the best germination percentage (14%). The pollen of *V. amygdalina* germinated in an in vitro medium containing 0.3-0.7g/l of boric acid. It can be seen that boric acid concentration induces the highest germination rate for *V. amygdalina* pollen tested as is represented by an optimum curve. The best germination medium contains 0.4g/l of boric acid with 22.02% of pollen germinated. Statistical analysis reveals that there is a significant difference ($p < 0.05$) between treatments with 0.4g/l boric acid concentration and other treatments except that with 0.5g/l. Statistical test also shows specifically that the difference in boric acid treatments in the germination of *V. calvoana* var. *calvoana* pollens ($p < 0.05$) can be observed at 0.4g/l boric acid with the best percentage germination of 13.44%. No significant difference ($p < 0.05$) between treatments is noticed on germination rates of *V. calvoana* var. *calvoana* with white florets. The best germination medium for *V. calvoana* var. *microcephala* is the one with 0.8g/l of boric acid that permitted 14.67% of pollen to germinate. It is the only treatment that differs statistically from the others.

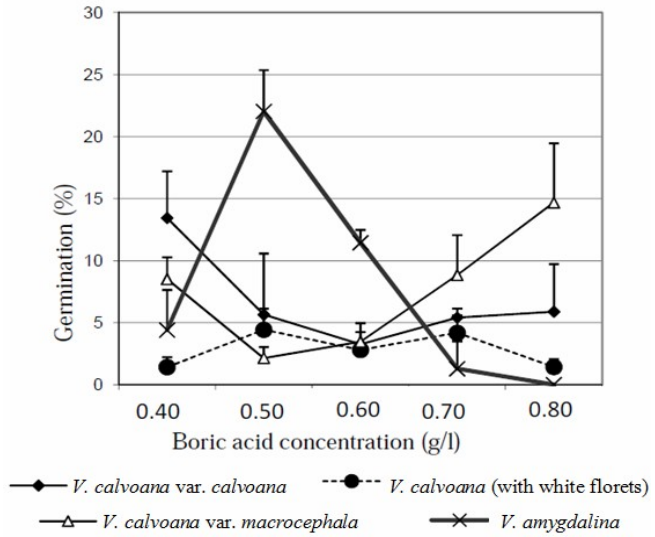

Fig. 6: Influence of boric acid concentration on germination capacity of four *Vernonia* pollen genotypes.

Influence of pH on pollen germination capacity

Pollen grains are sensitive to pH variations on the four genotypes studied (Fig. 7). The germination capacity of *V. calvoana* var. *calvoana* is optimal when the pH of the

culture medium is 5.6. Pollen of *V. calvoana* var. *calvoana* with white florets and *V. calvoana* var. *microcephala* has the optimum germination percentage at pH 5. The best germination rate is obtained at pH 4.6.

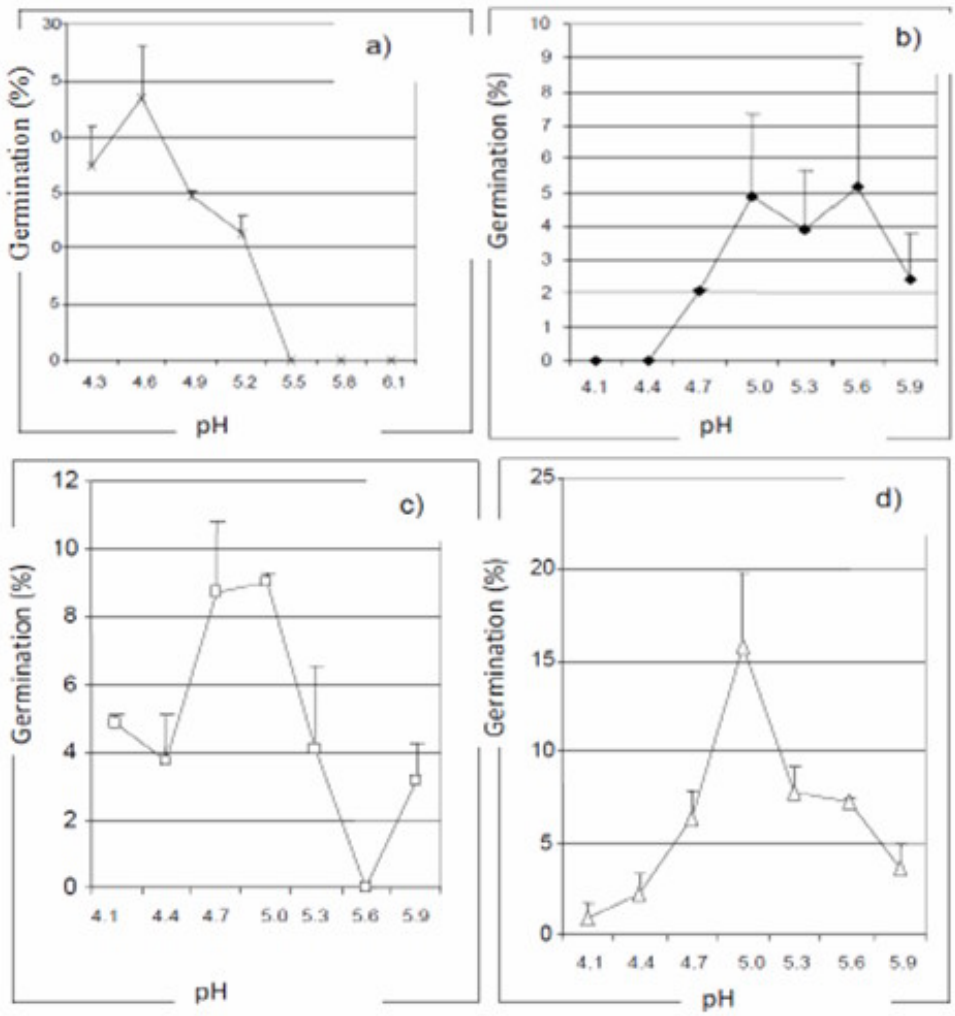

Fig. 7: Influence of pH on pollen germination capacity of *Vernonia* spp. a) *V. amygdalina*; b) *V. calvoana* var. *calvoana* with violet flowers; c) *V. calvoana* var. *calvoana* with white flowers; d) *V. calvoana* var. *microcephala.*

Discussion

Pollen morphology

Microscopic observations led to the conclusion that *Vernonia* pollen grains are spherical, porate, fenestrate and echinate with differences in sizes. This is proof of great variability in pollen morphology of *Vernonia* spp., which is in line with observations of Keeley and Jones (1979) presenting taxonomic importance. The pollen morphology thus provides evidence of the significant diversity observed in that genus indicating very large genetic resources for various species. It is also noticed that pollen of all those genotypes when shed out of anthers are coated with an adhesive and sticky white substance. This could be an adaptation to pollinators. In addition to an echinate character of pollen grains, this adhesive property facilitates the transport of the male gametophyte to the stigma by insects. It consolidates the fact that most angiosperms depend on animal pollinators for their sexual reproduction (Rossum et al., 2012). In fact, pollination depends on the matching between particular characteristics of flower (e.g. morphology, anthesis duration, scent and reward production) and pollinator morphology, as well as behavior (Amorin et al., 2012). Adhesive and echinate traits of pollen could be considered as an evolution trait which adapt the plant to pollinators, and favor cross pollination and gene mixture.

Effect of solution and sucrose concentration on pollen germination

The pollen grains of *Vernonia* spp. studied did not germinate in liquid medium with or without sucrose. Germination test succeeded only in solid medium (Fig. 8). It is suggested by Donfack et al. (2007) that the colloidal structure of agar which constitutes this medium helps the pollen with thin wall to regulate water exchange through the membrane. The agar limited the full entrance of water into the cell and preserved the pollen from physiological damages. *Vernonia* pollen germinates in high concentration of sucrose. It has also been shown that an improvement of pollen germination capacity is related to high sugar concentration especially in the presence of water, since the pollen is very water sensitive (Bair and Loomis, 1941). This relative sensitivity to water damage suggests that the species under study has a thin exine wall (Visser, 1955). In a small percentage of sucrose in the germination medium, high quantity of water enter through the membrane layer and causes loss of vital ions and other important soluble substances from the cell (Donfack et al., 2007). In this case, germination capacity is compromised. It was also observed that only the male gametophytes of *V. amygdalina* were able to germinate in BK medium and this could be due to its specificity. In the four taxa studied *V. amygdalina* is the only species with a difference.

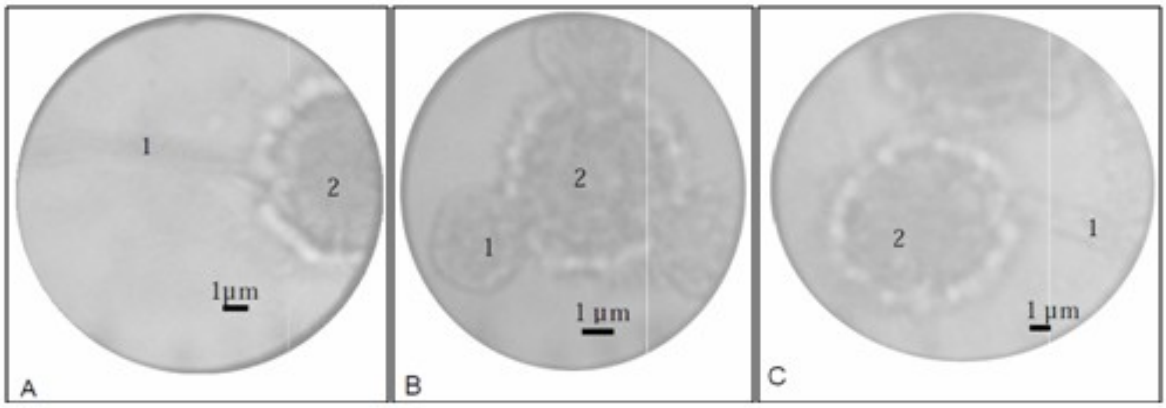

Fig. 8: Germinated pollen of *Vernonia* spp. A = Pollen of *V. calvoana* var. *calvoana* with pollen tube; B = *V. amygdalina* pollen at early stage of germination; C = Germinated pollen of *V. calvoana* var. *calvoana* with three pollen tubes; 1 = Pollen tube; 2 = Pollen.

Influence of temperature on pollen germination

Vernonia spp. produced pollen that is capable of germinating at up to 25°C and 35°C (Fig. 3). This could be considered as an important factor of adaptation of the plant to various climatic conditions and suggests that the appropriate time for pollination is during the day and not at night when temperatures are low. Flowers are built to fit their pollinators physically and to provide an appropriate reward (Judd et al., 1999). Pollination in that case should probably be done by specific pollinators which visit flowers by day. As discussed elsewhere (Bedford et al., 2012), the effect of recent climate change makes some areas even more climatically extreme, and this may impact the phenological overlap between plants and their pollinators (Vamosi et al., 2012).

Appropriate developmental stage for collecting pollen from *Vernonia* flowers

In the present study, step I is the best stage at which viable pollen could be collected from the anthers. At this stage, pollen is still protected by the anther and petals from external stress. The petals open and the pollen is pushed out of the anther by a plunger mechanism (Judd et al., 1999) as in step II. Moreover, it is better to use pollen collected within the anther to prevent infection, especially through spores, which grow faster than pollen tube and do not permit the observation of pollen grains in artificial media after 24 h.

Influence of storage duration on germination

The percentage of pollen germination decreases with the duration of storage at room conditions (Fig. 5). It was noted that, nine hours after collecting pollen, germination in the artificial media was unsuccessful. Thus *Vernonia* pollen grains have a very short life span. These pollen lives for six to nine hours when conserved within its capitulum at room conditions. A fragile exine wall could be the cause of the short life span of the male gametophyte. *Vernonia* pollen to be used in controlled pollination should be isolated in less than an hour from the plant for an optimum of viability of the male

gametophyte. In general, angiosperm pollen which loses viability very rapidly and can hardly germinate on artificial media is trinucleate (Pickert, 1988). When pollen is isolated from the anther, it loses its germination capacity three hours later. Three hypotheses can be developed to justify the short life span of pollen. The first is that climatic conditions change when the pollen is outside the anther. The second is that there could be microorganisms that infect and inhibit some functions or cause damage on the pollen structure and thirdly, the genetic facts could be involved. In addition, as demonstrated elsewhere (Siregar and Sweet, 2000), the importance of pollen isolation conditions from the anther is also proved.

Influence of boric acid concentration on *Vernonia* pollen germination

Boric acid concentration in the germination medium of *Vernonia* has an influence on pollen germination capacity (Fig. 6). The addition of 0.8g/L of boric acid in the germination medium of *V. calvoana* var. *microcephala* resulted in 14% germination and this was the best percentage. The effect of boric acid is increased by the elevation of temperature (Visser, 1955). The pollen of this genotype germinates best at 35ºC.

Influence of pH on germination capacity

Pollen grains were sensitive to pH variations in the four genotypes studied (Fig. 7). The germination capacity of *V. calvoana* var. *calvoana* is optimal when the culture medium is at pH 5.6. Pollen of *V. calvoana* var. *calvoana* with white florets and *V. calvoana* var. *microcephala* has their optimum germination percentage at pH 5. The best germination rate for *V. amygdalina* is obtained in a medium at pH 4.6. These results confirm that pH is an important factor which influences pollen germination depending on the species (Youmbi, 1993).

Pollination system

Investigations of the pollen morphology and physiology of the four *Vernonia* genotypes show that the pollen is matured at the opening of the petals (step I) when the stigma is not yet receptive. These mature pollen grains are then shed and pushed continually out by various developed hairs around the style through a plunger pollination mechanism as described in *Asteraceae* (Judd et al., 1999). It is also known that pollen has a life span of about six to nine hours. *Vernonia* spp. are thus protandrous. Cross pollination is therefore suitable in order to understand reproduction in these four genotypes but this does not mean that these plants are allogamous (Dierig and Thompson, 1993). It was also observed that the same capitulum contains many florets at different stages of development. It is possible for a stigma of one floret to be fertilized by pollen coming from another floret of the same inflorescence. The possibility for the first floret to be fertilized by pollen from another capitulum of the same plant is not to be rejected in advance. If research study has not proven the possibility of self incompatibility, however, two types of pollination, allogamy and autogamy are achievable.

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