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Assessment of the modes of pollen dispersal of *Vernonia amygdalina* Del. and *Vernonia calvoana* Hook

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The study of pollen dispersal and mode of fertilization of *Vernonia amygdalina* and *Vernonia calvoana* is a prerequisite to the understanding of genetic diversity and elaboration of improvement programs for the *Vernonia* genus. In this study, precise experimental designs were made for morphological and biological observations on *Vernonia* species capitulum in order to assess the distinctive effects of each of the three possible pollen transportation agents (insects, wind and rain water) on pollination. Results obtained show that the exclusive mode of pollen dispersal is entomophilous. Even though allogamy and autogamy are observed as two possible modes of fertilization in *Vernonia* spp., some arguments tend to favour allogamy. However, there is no clear cut position on the issue, hence the need for confirmation by further experimentation in controlled pollination. This study paves the way for the establishment of a genetic improvement program for this genus based on the results on pollen dispersal.

Key words: *Vernonia* species, allogamy, autogamy, pollination agent.

INTRODUCTION

Vernonia amygdalina Del., commonly called *Ndolé* or bitterleaf, and *Vernonia calvoana* Hook, known as sweet *Ndolé* or sweet bitterleaf, are the two tropical species of the Asteraceae family. *V. amygdalina* is a perennial shrub (Farombi and Owoeye, 2011), indigenous to tropical Africa occurring wild or cultivated all over sub-Saharan Africa (Igile et al., 1995; Oboh and Masodje, 2009). The leaves which generally have a bitter taste are eaten after crushing and washing to remove the bitterness. *V. amygdalina* is ubiquitous while *V. calvoana* has a more circumscribed habitat (Biholong, 1986). *V. amygdalina* is mainly found in secondary forests near villages or in

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fallows around forest zones and peri-forest savannahs in Cameroon (Biholong, 1986). *Vernonia* species is adapted to diverse climatic conditions. *V. amygdalina* is widespread in Africa, particularly in Cameroon, Benin, Togo, Senegal, and Côte d'Ivoire (Kalanda and Lisowski, 1995). *V. calvoana* is found in Camerron, Uganda, Kenya, Tanzania, Nigeria, and in Ethiopia (Biholong, 1986). In Cameroon (Nkambé, Nkonsamba, Buéa, Dschang, etc.), it grows near mountainous forests at about 2,000 m altitude. It is an annual plant but sometimes exhibits a perennial character depending on the nature of the soil. Plants aged two to three years with about 3 m of height can be found near streams (Fube and Njonga, 1987). As compared to *V*. *amygdalina*, its life span is shorter and its comestible leaves are less bitter.

Ndolé leaves are comestibles and exhibit antimicrobial, antihypertensive, antidiabetic, and laxative properties (Grubben and Denton, 2004; Dibong et al., 2011; Audu et al., 2012). In fact, all parts of *Vernonia* spp. are pharmacologically useful in phytomedicine (Oboh and Masodje, 2009; Farombi and Owoeye, 2011); roots and leaves are used to treat fever, hiccups, kidney disease, and stomach ache (Ngatu et al., 2014) in addition to their reported antihelmintic, antitumorigenic, and antimalarial properties (Hamowia and Saffaf, 1994; Abosi and Raserika, 2003; Izevbigie et al., 2004; Oboh and Masodje, 2009). Akah and Okafor (1992), Nwanjo (2005), and Atangwho et al. (2010) also reported hypoglycaemic and hypolipidaemic effects of *Ndolé* leaf extracts in experimental animals. Moreover, Oboh and Masodje (2009) and Udochukwu et al. (2015) revealed the moisture, protein, ash and mineral content and antimicrobial effects of bitter leaf leaves. Recently, a comprehensive review done on the medicinal potentials of plants of the genus *Vernonia* (Toyang and Verpoorte, 2013) also highlighted most of the aforementioned useful properties of bitter leaf.

These are probably the main reasons why *V. amygdalina* is much consumed all over sub-Saharan Africa and as exotic food in some parts of Europe and America. In sub-Saharan Africa especially in Central and West Africa, *Ndolé* constitutes a much appreciated delicacy in homes and modern restaurants. Also, the number of African restaurants is on a rapid increase in France and other European countries (Le Roux, 1996; Defrance, 1997). Meanwhile, the demand for exotic food products like *Ndolé* is on the rise in Europe (Volatier, 1997; Gillet, 1997) where the market for such foods currently represents over 10 billion of CFA Francs (Bidima and Voufo, 2007). For instance, the sales of *Vernonia* (*Ndolé*) leaves as vegetable is strongly developed in France and Belgium where there are big Cameroonian communities (Bidima and Voufo, 2007; Tabuna, 2000). In France, for example, about 37% of consumers buy exotic products (Gillet, 1997; Volatier, 1997). The sales of *Ndolé* are extending to other European countries like the United Kingdom, Germany and Switzerland which constitute a potential important market outlet (Tabuna, 2000).

Due to the importance of *Ndolé*, it is important for plant breeders to master the production and improvement procedures. In addition to having important medicinal properties, comestible *Vernonia* can play a role in food safety. More and more, it is being cultivated by gardeners in subsistence agriculture in rural areas and marshy zones especially in urban areas. However, this is done without any well-conceived genetic improvement program in place. In fact, the realization of such a program is feasible through a better understanding of the reproductive biology of the plant. In this regard, Judd et al. (1999) believe that Asteraceae in general are allogamous and that their pollen dispersal is anemophilous, entomophilous, and hydrophilous. Concerning the mode of fertilization, the flower of *Vernonia* is considered to exhibits protandry like other Asteraceae in general (Perry and Hirons, 1967; Tyler and Arthur, 2006; Guerrina et al., 2016). This situation naturally imposes allogamy on this plant. However, it is observed that the flowers of the same capitulum do not open at the same time, giving the possibility of self-fertilization between flowers of the same capitulum and hence autogamy. With regards to the floral morphology, it seems not appropriate to conclude on the different modes of pollen dispersal given that water which is obviously subject to gravity will not easily transport pollen located under the stigma to fertilize it. These observations led us to put in place a protocol to investigate the modes of pollen dispersal of the two *Vernonia* spp. in this study.

MATERIALS AND METHODS

Plant material and study site

Plant materials used for this study were two species of *Vernonia*: *V. amygdalina* and *V. calvoana*. The study was conducted in Mfou in the Centre Region of Cameroon, from November 2013 to February in 2014. Mfou is the head quarter of Mefou-and-Afamba Division, located at 4°27'00'' latitude north and 11°38'00'' longitude east. The Mfou zone is characterized by an equatorial Guinean climate with four seasons: a long rainy season from mid-August to mid-November, a long dry season from mid-November to mid-March, a short rainy season from mid-March to mid-June, and a short dry season from mid-June to mid-August. Average daily temperature varies from 23 to 24°C, and annual rainfall is 1,600 to 1,800 mm. The soil of this site is ferralitic, acid and is strongly desaturated with ruddle aspect, presenting a concrete horizon that protrudes on hill tops. Nine (9) plants of *V. amygdalina* were chosen at random from the fallow, while nine plants of *V. calvoana* were planted at 5 m interspacing, after clearing a surface area of 50 m^2. The study site was a two-year old fallow dominated by *Chromolaena odorata* (L.).

The experimental designs (Figures 1, 2, and 3) were put in place once the first floral bud appeared. It was considered that the agent being tested (wind, rain water or insects) influenced pollination if at the end of the process the achene obtained germinated. Seeds were put in envelopes, labeled, and conserved in the same non humid conditions. Germination test was done on the seeds obtained from field experiments in the laboratory of Biotechnology

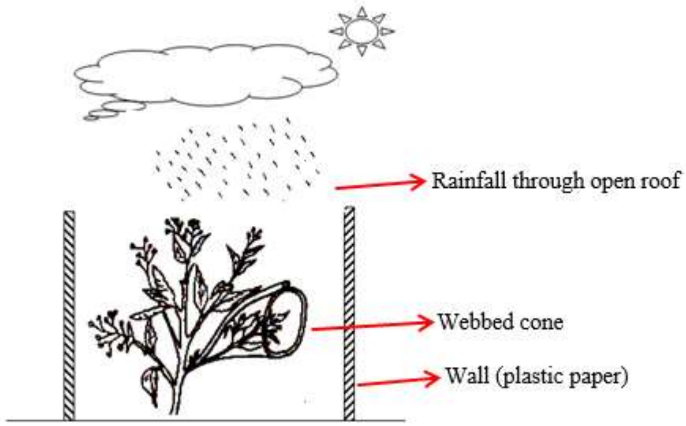

Figure 1. Design to study the role of rain water in dispersal of *Vernonia* spp. pollen.

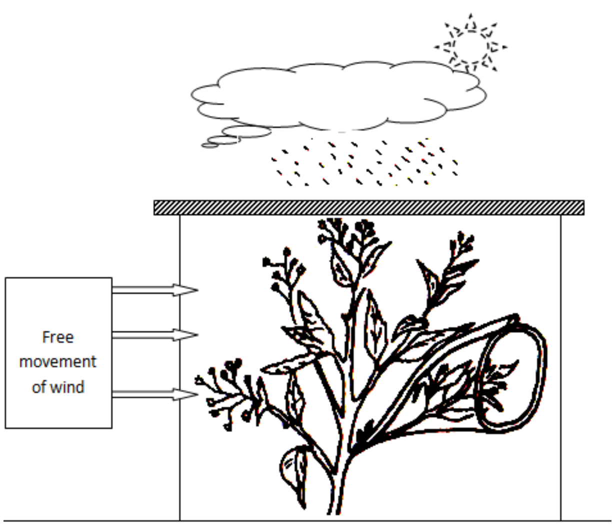

Figure 2. Experimental design to study the effect of wind on dispersal of *Vernonia* spp. pollen.

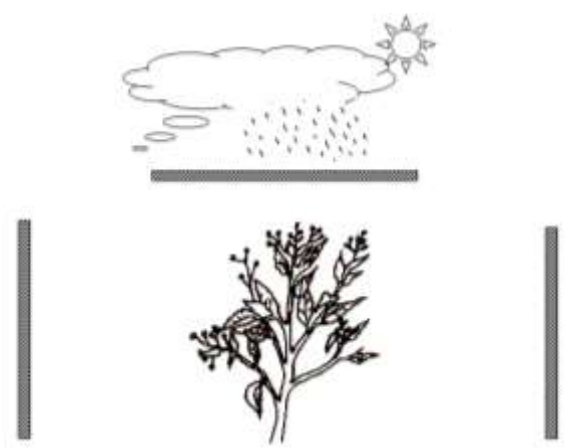

Figure 3. Experimental design to put into evidence the role of insects on dispersal of *Vernonia* spp. pollen.

and Environment of the University of Yaounde I. The seeds were germinated in Petri dishes on cotton soaked with tap water.

Experimental design to limit insects and wind

This design was established to determine the role of rain water in pollen dispersal. For the construction of the fence (Figure 1), four poles, each 3 m long, and higher than the *Vernonia* plant were placed at the four corners around the plant. The four walls were covered with plastic transparent paper while the roof was left open to rainfall. Three distinct fences around three different plants were constructed for each of the two *Vernonia* spp. A webbed cone was constructed using iron wires covered with impregnated mosquito net. An adhesive tape was used to hold the pointed end of the cone on the branch of *Vernonia* under study (Figure 1).

Three different branches of the same plant were isolated with the webbed cones. For each species of *Vernonia*, nine branches belonging to three plants (three branches per plant) were isolated to prevent insect visits. The aim of this design was to avoid the action of wind and insects on the pollination process while maintaining the action of rain water which could fall through the open roof.

Experimental design to limit insects and rain water

This design was devised to analyze the role of wind as pollen dispersal agent. Eight wooden poles were used to construct a shed with open walls (Figure 2) around the plant. The roof was covered with a transparent plastic paper to avoid rainfall on the plant. For each species of *Vernonia*, three sheds were constructed over three different plants. Three branches per plant with flowers were protected with webbed cones against insect pollination. The goal here was to exclude the action of rain water and insects in the process of pollination while preserving only the action of wind.

Experimental design to determine the role of insects as pollen dispersal agents

This third design helped to avoid the action of rain water in pollen dispersal by using a transparent plastic tissue over the plant, supported by four wooden poles. Another barrier was created on the four walls around the plant to screen it from wind flow. A 2 m space was allowed between the plastic roof and the upper edge of the walls to permit insect visitation of the plant (Figure 3). Six plants were used in this experiment; three per species. On each plant, three flowered branches were isolated using the webbed cones (Figure 3). All these plants were exposed to wind and rain during the field experimentation period. At the end of the process, pollen grains from insect-isolated and non-isolated branches were harvested from the plants. The non-isolated grains constituted the control treatment of the entire experiment.

Data analysis

Data obtained on the germination percentage of pollen grains of the two *Vernonia* spp. studied was treated by analysis of variance (ANOVA) with the Minitab version 16 software (Ryan et al., 2004). Comparison and classification of means was done using Student-Newman-Keuls test at the threshold of 5%. The ANOVA was performed with data after ensuring conformity of these data with ANOVA assumptions (normality and homogeneity of variance). Non-compliant data to the ANOVA assumptions were analyzed by the Kruskal-Wallis test and averages were compared by the Mann-Whitney U test at the threshold of 5% using IBM SPSS software.

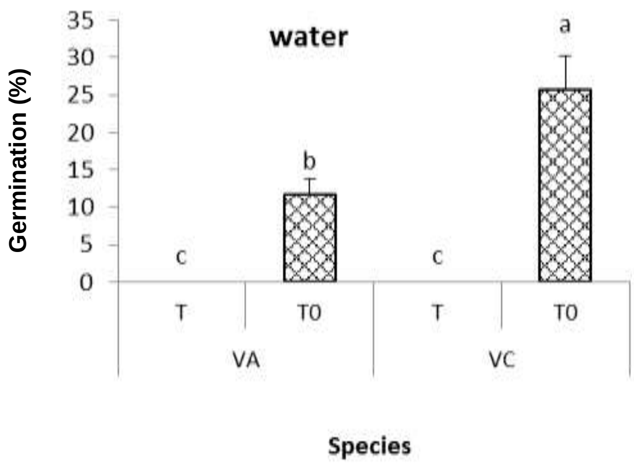

Figure 4. Germination percentages of two *Vernonia* species in the presence of rain water and the absence of wind and insects. T: Absence of wind and insects, presence of water; T0: presence of wind, insects, and water; VA :*Vernonia amygdalina*, VC: *Vernonia calvoana*; Values followed by the same letter are not significantly different at the threshold of 5% with the Student-Newman-Keuls test.

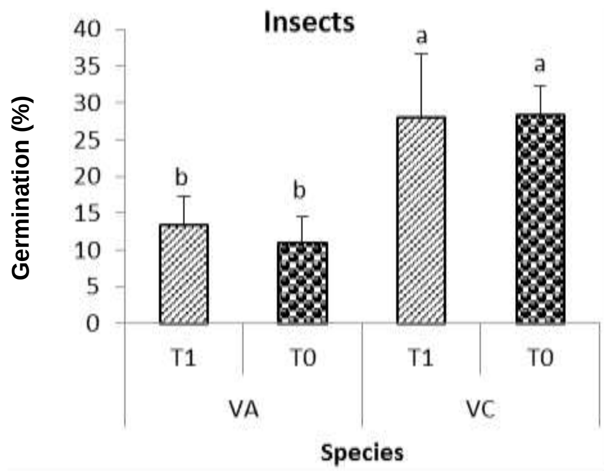

Figure 6. Percentage germination of pollen from the two species of *Vernonia* sp. in the presence of insects and in the absence of water and wind. T1: Absence of water and wind, presence of insects; T0: presence of wind, insects and water; VA: *Vernonia amygdalina*; VC: *Vernonia calvoana*; Values followed by the same letter are not significantly different at the threshold of 5% with the Student-Newman-Keuls test.

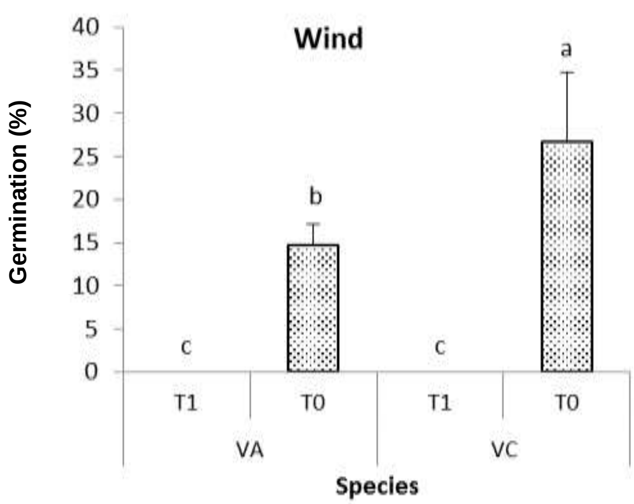

Figure 5. Percentage germination of two *Vernonia* species in the presence of wind and in the absence of water and insects. T1: Absence of water and insects, presence of wind; T0: presence of wind, insects, and water; VA: *Vernonia amygdalina*, VC: *Vernonia calvoana*; Values followed by the same letter are not significantly different at the threshold of 5% with the Student-Newman-Keuls test.

RESULTS

Mode of pollen dispersal

Pollen dispersal by rain water

Figure 4 presents the germination percentages of pollen grains of *V. amygdalina* and *V. calvoana* resulting from plants grown in the absence of wind and insects. Results show that the pollen germination percentage of control (wind + water + insects) is 11.67% for *V. amygdalina* and 25.67% for *V. calvoana*, as against 0% for the treatment (rain water only) on the two species. No germination was observed in the treatment (Figure 4) indicating that water is probably not the agent of *Vernonia* pollen dispersal.

Pollen dispersal by wind

Results of germination (%) of pollen grains of *Vernonia amygdalina* and *V. calvoana* in the absence of water and insects are shown in Figure 5. It can be observed (Figure 5) that percentage germination of pollen of the control (wind + rain water + insects) is 14.67% for *V. amygdalina* and 26.67% for *V. calvoana*. The percentage germination of pollen from the treatment (wind alone) is 0% for both species (Figure 5). The fact that no germination was observed in the treatment (wind) indicates that wind is not the pollen dispersal agent in *Vernonia* spp.

Pollen dispersal by insects

The germination percentages of pollen from *V. amygdalina* and *V. calvoana* in the absence of water and wind are illustrated in Figure 6. The germination percentages of pollen from the control (wind + water + insects) for *V. amygdalina* (11.00%) and *V. calvoana* (28.33%) are statistically identical to those of the treatment (insects only): *V*. *amygdalina* (13.33%) and *V. calvoana* (28.22%). This observation shows that insects are responsible for the dispersal of pollen grains of *Vernonia* spp.

Possible mode of fertilization of Vernonia spp.

Based on morphological analysis of *Vernonia* spp, flowers and pollen morphology (Nguimkeng et al., 2015), and on experimentation done on the mode of pollen dispersal of the two *Vernonia* spp. studied, it can be hypothesized as is currently admitted that fertilization in *Vernonia* spp. is done by allogamy. Mindful of the fact that the flowers of the same capitulum do not open at the same time in this genus, a second hypothesis could be that fertilization in *Vernonia* spp. is done by autogamy between florets of the same capitulum. However, it is necessary to verify these hypotheses by further thorough experimentation. In this study, observations made on the capitulum of *Vernonia* spp. show that the florets of the same capitulum are not of the same age and are found to be at different developmental stages. Central florets are younger than those on the peripheries. The expulsion of pollen grains out of the anthers by plunger pollination mechanism is a common procedure in Asteraceae. By this mechanism, pollen grains are released out of the anther lobes by the style which progressively scrapes pollen grains as it grows, to put them at the disposal of pollinators.

During the vertical elongation of the style, it progressively opens horizontally along a median axis to form two symmetrical ligulate parts folded outward. At maturity, the upper surface bears the stigma while the lower surface is covered by pollen grains. Analysis of pollen morphology and mode of pollen dispersal helped to hypothesize on the possible allogamous mode of fertilization in *V. amygdalina* and *V. calvoana.* However, there is need to experimentally test the mode of fertilization in order to confirm this hypothesis. This could be done by examining seed germination upon controlled self-pollination and controlled cross-pollination of *Vernonia* spp. flowers.

The second hypothesis which stipulates that autogamy is equally possible in *Vernonia* spp. was also considered probable (Figure 7). It is therefore possible that fertilization in *Vernonia* spp. is done both by allogamy and autogamy.

DISCUSSION

The results obtained from this work have put into evidence the existence of a single mode of pollen transport in natural milieu for the two species studied (Figure 6). In fact, 11.00% germination of pollen grains was obtained for *V. amygdalina* and 28.33% germination for *V. calvoana* when the flowers were exposed to pollination agents in natural milieu. These results corroborate those of Judd et al. (1999) and Grubben and Denton (2004) who observed that pollination of *Vernonia* is entomophilous. However, our results are contrary to those of the same authors who declared that pollination in Asteraceae is equally hydrophilous and anemophilous.

The results of our experimentation clearly demonstrated that in the case of *Vernonia,* pollination is exclusively entomophilous (by insects). Nguimkeng et al. (2015) believe that pollen morphology of *Vernonia* adapts this genus to this mode of pollen transfer since it is echinulate and covered by a substance which sticks it easily to the pollinator. The presence of this same gelatinous sticky substance does not facilitate an anemophilous mode of pollen transport since there is need of more force to unstuck the pollen from the style given their tiny sizes. Rain water does not also constitute a medium of pollen transfer in *Vernonia* spp. This is because pollen formed at the end of floret maturation process is located under the stigma. The law of gravity imposes that this male gametophyte located under the stigma be carried by rain water downwards and not up over to the stigma located on the part above it.

The observations by Nguimkeng et al. (2015) suggest that fertilization in *Vernonia* spp. is possibly allogamous (Figure 7). Perry and Hirons (1967) and Judd et al. (1999) suggested this mode of fertilization in Asteraceae in general, on the basis of observations done in natural milieu. *Vernonia* flowers exhibit the phenomenon of protandry which is naturally proper to allogamy since, pollen from a flower attains maturity when the gynoecium is not yet matured. In such conditions, if the life span of this male organ is short as is the case with this species, the pollen is destined to obviously pollinate the gynoecium of another matured flower.

Based on observations in this study (Figure 7), it could be considered that allogamy is a mode of fertilization in *Vernonia* spp. even though autogamy is not to be excluded as a mode of fertilization as long as evidence of incompatibility has not been established. Even though the flowers exhibit protandry, those of the same capitulum do not mature at the same time. The central florets are younger than those at the periphery. It is therefore probable that pollen of central florets fertilize the peripheral florets whose gynoecia have attained maturity. In this case, we assume autogamy since the florets of the same capitulum result from the same genetic material. Another possible consideration is the fertilization of a floret belonging to a different capitulum of the same plant as illustrated in Figure 7. Further detailed studies of *Vernonia* spp. on the flower lifespan, exact stage and time (hours of the day) of pollen release, stigma receptivity, proper pollen storage and germination conditions (Monteiro et al., 2015) are recommended. These considerations are valuable for eventual establishment of an efficient regeneration system through

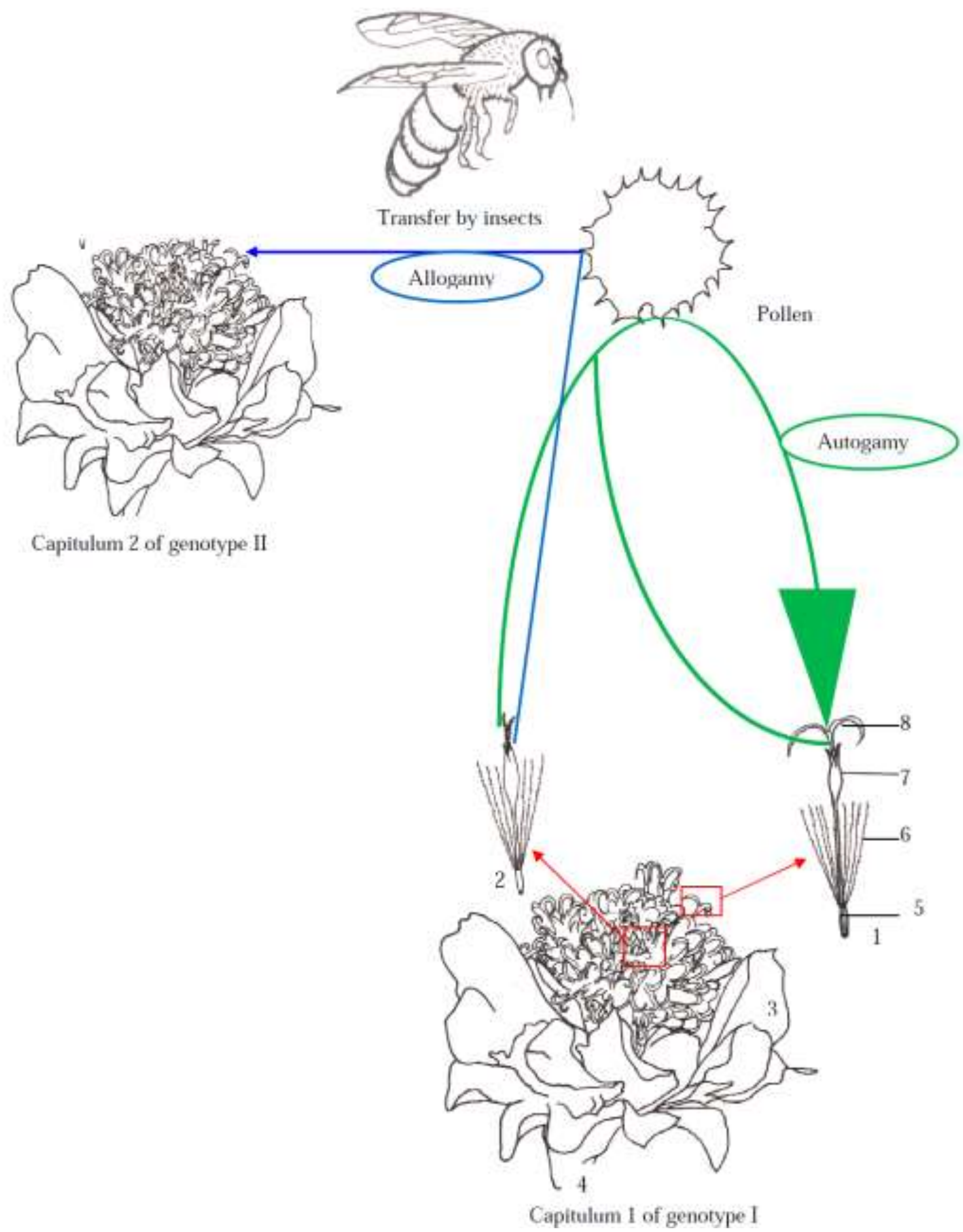

Figure 7. Possible model of pollination system in *Vernonia amydalina* and *Vernonia calvoana.* 1: Matured peripheral floret; 2: young central floret; 3: receptacle of capitulum; 4: peduncle of capitulum; 5: receptacle of floret; 6: aigrette; 7: petal; 8: stigma.

classical breeding or micropropagation (Min et al., 2015) for *Vernonia* spp. cultivation.

Conclusion

Results obtained showed that the exclusive mode of pollen dispersal of *V. amygdalina* and *V. calvoana* was entomophilous. Based on observations and pending thorough experimentation, fertilization in the two species can either be allogamous or autogamous with allogamy more preferential. It was found that *V. calvoana* pollen germinates better than that of *V. amygdalina.* Information obtained on the mode of pollen dispersal and fertilization in *Vernonia* spp. could be exploited for genetic improvement of this important plant. It could also help to understand the genetic diversity within the genus and the preponderant role of insects in the preservation of biodiversity.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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