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In vitro pollen germination of Neelakurunji - a semelparous plant species

P. Jayaprakash*, John Peter, R. Nisha, V.K. Vikas, and M. Sivasamy

ICAR- Indian Agricultural Research Institute, Regional Station, Wellington -643 231, The Nilgiris, Tamil Nadu, India

ABSTRACT

The Neelakurunji (Strobilanthes kunthianus) is a semelparous plant species that flowers once every 12 years in the Western Ghats of South India. A pollen germination medium to germinate kurunji pollen in vitro was developed and reported for the first time. The pollen germination medium developed by Brewbaker and Kwack medium was modified; urea and multivitamin were added to develop a medium supporting more than 91% pollen germination in vitro. Kurunji pollen is elliptical, and the pollen tubes peculiarly emerge from the belly of the pollen. This new pollen germination medium (PGM) consisted of 15% sucrose +15% PEG 4000 + 100 mg per L boric acid + 450 mg per L calcium nitrate +75 mg per L potassium nitrate+ 150 mg per L magnesium nitrate+ 50 mg per L urea+200 mg per L Neurobion forte(multivitamin tablet). In addition, urea has been added as one of the constituents to the pollen germination medium for the first time. Also, the pollen viability and stigma receptivity were tested.

Keywords: *Strobilanthes kuntianus*, *In vitro* pollen germination, Urea, Multivitamin, Pollen morpology.

INTRODUCTION

Strobilanthes kunthianus (Nees) T. Anders. (Family *Acanthaceae*) known as 'Neelakurinji' is an endemic shrub that generally grows above 1,800 m commonly located in the sholas of the Western Ghats (Jomy ,9). It normally reaches a height of 30 to 60 cm and can grow beyond 180 cm under favorable conditions and is considered as a major component of the tropical evergreen forest. Wood, (13) reported considerable variation in the life history and mass-flowering of *Strobilanthes*. One of the oldest mountain ranges, The Nilgiris which literally means Blue Mountains has derived its name from the gregarious blossoms of purplish-blue flowers of Neelakurinji.

Several species of *Strobilanthes* (*Acanthaceae*) has been reported to be distributed in the Indian subcontinent and South-East Asia which are also semelparous and flower at intervals of 3–12 years. The frequency of flowering of successive generations in such species is highly variable. Based on the records of one of the earliest European families settled in the hills, the flowering periodicity of this species has been well documented which shows a 12-year flowering cycle from the Nilgiri Hills between 1838 and 1934. Similarly, a 12-year flowering cycle for this species has also been reported in the Palani Hills. Recently, *Strobilanthes kunthianus* flowered during December, 2018- February, 2019 (Fig. 1), covering the hills with a bluish purple carpet from its dense massive bloom, attracting a large number of tourists.

*Corresponding author: jpsarit@gmail.com

Generally, *Strobilanthes* spp. takes 12 years to flower (Anitha and Prasad,3). Sharma *et al.* (12) reported that this species is semelparous or monocarpic and are perennial flowering plants that flower once in their lifetime and die. The term plietesial is used to refer to these perennial monocarpic plants. Plietesial life history includes gregarious flowering, supra-annual synchronized semelparity and seed setting. Due to the semelparous nature of these plants, they have only one chance to reproduce and hence they are committed to a reproductive event at a specific time regardless of the environmental conditions.

In general, semelparous species invest all their resources into seeds and generally produce a larger seed crop which is referred to as mast seeding. A long-lived gregariously flowering semelparous plant is even more committed to this single event because all of its kin will flower and die at the same time. Therefore in order to facilitate reproductive assurance, these semelparous species devise a major reproductive strategy. Semelpary is found in a wide range of plant species covering at least 20 families (Young & Augspurger, ,14).

Controlled pollinations on *S. kunthianus* clearly showed that the species is self-compatible. Fruit and seed set were reported to be very high, even under open-pollination conditions. Self-compatibility seems to have adaptive significance in mass seeding of this species. Although reproductive synchrony, a characteristic feature of semelparous species is known to enhance the plant's ability to attract pollinators and the large number of fresh flowers available on each plant results in a high degree of geitonogamy.

Fig. 1: Photomicrograph showing the (A) Neelakurunji in full bloom in 2018 (B) Kurunji bloom at IARI Garden, Wellington (C) Kalkurunji

Cross pollination is favored by several cumulative floral traits such as the life of the flower extending for 2 days with adequate floral rewards and stigma receptivity, sensitive stigma to expose the receptive surface to the path of the incoming pollinator and away from the outgoing pollinator. Also dehiscence of anthers in response to the pollinator's visit, and self-compatibility has enabled *S. kunthianus* to achieve the mast seeding habit.

The reproductive success of a species depends on the pollen viability and the efficiency of pollen transfer. Successful fertilization depends on the fertility and viability of the pollen grains. Keeping this in view, a first and foremost effort was made to develop *in vitro* pollen germination medium for *S. kunthianus* to study the metabolism, nutritional requirement and other growth characteristics which is otherwise impossible due to the presence of tissues of stigma and style and other complex metabolites.

MATERIALS AND METHOD

The study was conducted at ICAR-Indian Agricultural Research Institute, Regional station, Wellington, The Nilgiris, Tamil Nadu, situated at 11°N latitude and 77° E longitude at 1850 MSL during August 2018 to February 2019. Pollen samples were collected from the Neelakurunji flowered at IARI Wellington and nearby hills. Another species

commonly called as Kal kurunji, *Strobilanthes wightianus* has also flowered and was included for this study *(Fig.1).* The authenticities of these species were confirmed with Botanical Survey of India at Coimbatore with fresh specimens.

Flower buds were collected from the flower early in the morning. Once anther started dehiscence, the pollen was dusted directly on to the germination medium by gentle tapping and cultured according to the pollen germination media (PGM) droplet method (Jayaprakash *et al.* (7). All observations on pollen germination and pollen tube growth were recorded 15 minutes after incubation at 17±1°C. A minimum of 250 pollen grains were counted from 5-10 random fields. Incubation temperature was maintained from 18 to 28°C. Initially one of the key media was selected and the constituents were altered according to the germination percentage of the pollens. The best three media from each treatment were selected for observation.

Solution of different concentrations of sucrose (1-50%), boric acid (25-500 mgl^{-1}), calcium nitrate, potassium nitrate and magnesium sulphate (50- 500 mgl^{-1}) were prepared. Brewbaker and Kwack(4) (BK) medium was used (10% sucrose, 100 mg mgl^{-1} boric acid, 300 mgl^{-1}calcium nitrate, 200 mgl^{-1}magnesium sulphate and 100 mgl^{-1} potassium nitrate) as the base medium for pollen germination experiments. The salts of BK medium were varied after finalizing the concentration of osmaticum.

In addition to above media constituents, urea(10-100 mgl^{-1}) and multivitamin Neurobion *forte* (10-300mgl^{-1}) was added to control pollen tube burst. Each tablet of Neurobion forte contains (Thiamin mononitrare(B1) 10 mg+Riboflavine(B6) 10 mg + Cyanocobalamin(B12)15µg + Nicotinamide(B3) 45mg + Calcium pantothenate (B5) 50 mg. Neurobion *forte* tablet manufactured by Procter and Gamble Health Ltd, Goa, India has been used for pollen germination experiments

Fluorescein diacetate (FDA) (Heslop-Harrison and Heslop-Harrison,5) staining method was used to assess viability. The fluorochrome FDA scored pollen grains that fluoresced brightly as viable and others as non-viable.

Stigma receptivity of kurunji flowers was tested by Alexander staining (2) method which does not require a fluorescence microscope. Appropriate flower buds were emasculated and pollinated with fresh pollen at different time intervals. The pollinated flowers were collected and fixed in acetic alcohol after 1 h. Then stigmas were transferred to equal quantity of staining and clearing mixtures, gently heated over a flame and mounted on a drop of stain for observation.

During the evaluation using scanning electron microscopy (SEM), properly dried pollen of Neelakurunji was placed onto transparent double-sided tape on the disc surface of polished aluminum stubs. The sample on each stub was sputter-coated with a gold layer c. 200 Å thick. Pollen grains were observed in a Stereoscan 360 SEM, operating at 10 kV and photographed at different magnification.. The length (L) and the width (W)of grain, L/W ratio, distance between two furrows and the width of ridges in elliptical pollen and also the base, the altitude, and the width of the grain and ridges in triangular pollen were measured. Four randomly selected pollen grains were observed and damaged or irregularly positioned grains were excluded from observation.

RESULTS AND DISCUSSION

The germination ability and the growth of pollen tube are an important sign of compatible pollination. The viability of kurunji pollen may be tested with FDA stain. *In vitro* pollen germination is a good way to determine germination percentage, but the main problem lies in the detection of suitable media for each cultivar. Pollen germination and growth of pollen tubes are important research materials for morphological, physiological, biotechnological, ecological, evolutional, biochemical and molecular biological studies. A pollen grain was considered as germinated, if the pollen tube length becomes at least twice greater than the diameter of the pollen grains. After pollination with compatible pollen, only the fastest growing, most vigorous pollen grain will result in fertilization.

PGM droplet technique was used to study the pollen germination in *S. kunthianus.* In a petridish, a droplet of pollen germination medium was placed using a glass rod. Drops of many different media could be placed within few centimeters between them. This could aid in the testing of more number of media in a short period. Initially basic BK salts with different concentration of sucrose was tested. Pollen grains start to germinate at 10% sucrose solution showing 23.57% germination along with 16.58 µm tube length, while maximum 44.59% pollen germination along with 16.77 µm long pollen tube development occurred in 15% sucrose solution. But, 36.75% pollen germination occurred with 27.86 µm long pollen tube development in 18% sucrose solution. (Fig.2*).*Invariably in all media pollen and pollen tube bursting was seen. Hence sucrose concentration of 15 percent was fixed and other salts were varied one by one to arrive an optimum medium. Sucrose acts as respiratory substrate for pollen grains as well as important to maintain the regulation of osmotic pressure. Invariably, sucrose has been used as

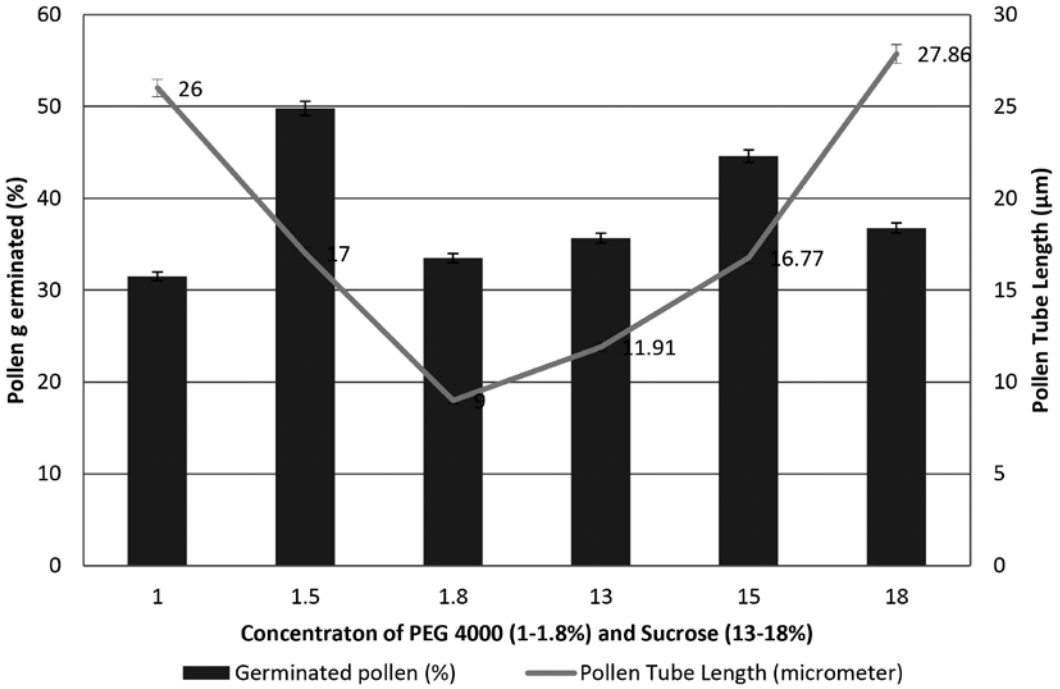

Fig. 2: Effect of Poly ethylene glycol (PEG) and sucrose on pollen germination and pollen tube length of neelakurinji *In vitro*

carbon source in most *in vitro* pollen germination medium (Brewbaker and Kwack 4; Jayaprakash and Sarla.6; Jayaprakash *et al.* 8)

Having fixed sucrose at 15%, PEG 4000 concentration varied from 10 to 18 percent. Individually, 15 percent PEG 4000 showed 49.79% pollen germination with the pollen tube length of 17.00 µm (Fig. 2). Whereas at higher concentration of 18% PEG pollen germination of 33.47% along with 9.78 µm length of pollen tube was observed. At low concentration of 10% PEG, pollen germination was 31.51 per cent and pollen tube length was 26.18 µm. Addition of poly ethylene glycol maintained the osmaticum and reduced pollen burst in most species for example in pigeonpea (Jayaprakash and Sarla,6), eggplant and its wild species (Jayaprakash *et al.* 8) etc**.** PEG is also believed to regulate the permeability and stability of plasma membrane. The inhibitory effects of boric acid could also be overcome by the supplementation of PEG . The pollen tubes were long and narrow without any deformities. Presence of regular callose indicated that addition of PEG to the medium remarkably improved the germination and growth of the pollen tube.

Keeping the sucrose concentration at 15 per cent and PEG 4000 at 15 percent, the boric acid levels were altered. At 100 mgl^{-1} boric acid showed 53.36 % germination along with 16.63 µm long pollen tubes (Fig. 3). Below or above this concentration, the pollen germination as well as the pollen tube length is less. Inclusion of boric acid to the medium stimulated the pollen germination along with the growth of the pollen tube once osmaticum has been stabilized with sucrose and PEG. The stimulating effect of boron on pollen germination and pollen tube growth was discovered as early in 1933. Boron is also found in style and stigma to enhance sugar uptake and play a key role in pectin synthesis in the growing pollen tubes. The viability of the pollen, pollen germination and pollen tube growth may be affected if the medium is not supplemented with boron. Initially, tube bursting occurred in the medium consisting of sucrose. According to the reports of Acar *et al.* (1) tube bursting occur due to elimination of boric acid from pollen culture medium. Thus, boron plays a significant role in fertilization of flowering plants towards successful seed production

Among the salts, calcium nitrate is another important one for pollen germination. Media with sucrose 15% + PEG 4000 15% +100 mgl^{-1} boric acid having different levels of calcium nitrate was tested. At an optimum concentration of 300 mgl^{-1}, the highest pollen germination of 57.89% was observed with a pollen tube length of 11.13 µm. The pollen germination was 36.3 and 42.96 percent at calcium nitrate concentration of 200 and 400 mgl^{-1} respectively. Nearly, 50% germination of the pollens occurred when the medium was supplemented with calcium nitrate (Fig. 3). Thus the results indicate that

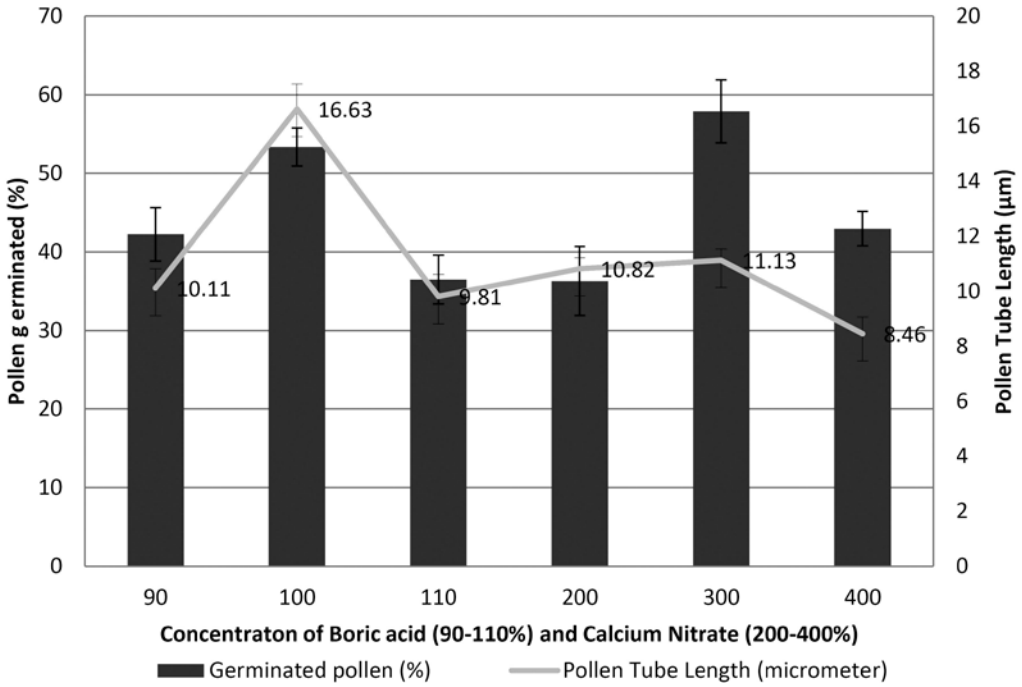

Fig. 3: Effect of Boric acid and Calcium Nitrate on pollen germination and pollen tube length of neelakurinji

calcium nitrate was the most effective one. Calcium is one of the most important cation which is involved in pollen germination as well as pollen tube growth. Calcium plays an important role in maintaining the membrane integrity and permeability (Brewbaker and Kwack, 4) and it also plays a significant role in pollen tube growth and pollen tube development.

In addition to above media having optimum osmoticum and other salts fixed, a small quantity of nitrogen source in the form of urea was added to enhance pollen germination of neelakurunji. At 50 mg/l concentration pollen germination was 59.13% with the pollen tube length of 17.78 µm (Fig. 4). Urea has so far been used as a nitrogen source in culture medium of either bacteria or fungal (Taabodi *et al.* 10; Osama *et al.* 11), and rooting medium. This is for the first time urea was used in pollen germination medium

The strength of magnesium sulfate and potassium nitrate were varied next. At 3/4th strength of these salts, a maximum of 61.37% pollen germination with 15.83 µm pollen tube length(PTL) was observed whereas at 50% concentration it was 46.03 per cent pollen germination with 7.5 µm pollen tube length. Potassium nitrate and Magnesium sulphate were also reported to supplement the tube growth as reported in case of *in vitro* pollen germination of *Saccharum* sp. KNO_3 has also been reported to regulate the osmotic potential for the swelling of pollen grains.

In order to improve pollen germination further, various multivitamins tablets which were available in Pharmacy have been tested. Among them, Neurobion forte showed effect on pollen germination and pollen tube growth. This medium with sucrose 15% + PEG 4000 15% + 100 mgl^{-1} boric acid + 300 mgl^{-1} calcium nitrate + 50 mgl^{-1} urea was supplemented with 20 0mg of multi-vitamin (neurobion) showed 62.74% germination with 21.41 µm pollen tube length (Fig. 4). Zhang *et al.* (15) found that the vitamin B1 enhanced the pollen germination in rice and suggested the uclacyanin gene, *OsUCL8* may be linked to production and regulation of vitamin B1 in rice pollen. The B-complex vitamin used in this study contains VB3, VB6, VB12, VB5 besides VB1. The role of which B vitamin sub unit enhances kurunji pollen germination may be assessed in its next bloom, i.e. 2030. In Western ghats of India Neelankurunji generally flowers once in 12 years and its regular flowering has been well documented since 1838 to 2018. However, it is likely that it may flower in some habitats in a year before or later. With further slight modifications in calcium nitrate at 450 mgl^{-1}, highest germinating pollen (91.46%) along with 47.424 µm long pollen tube developed whereas the pollen germination was 83.73 and 74.56% with a pollen tube length of 25.24 and 27.12 µm of 420 and 480 mgl^{-1} of calcium nitrate respectively (Fig. 5).

Pollen viability test with FDA stain differentiated the viable and sterile pollens and the results matched with *in vitro* pollen germination observations. FDA fluorochrome clearly demarcated between viable

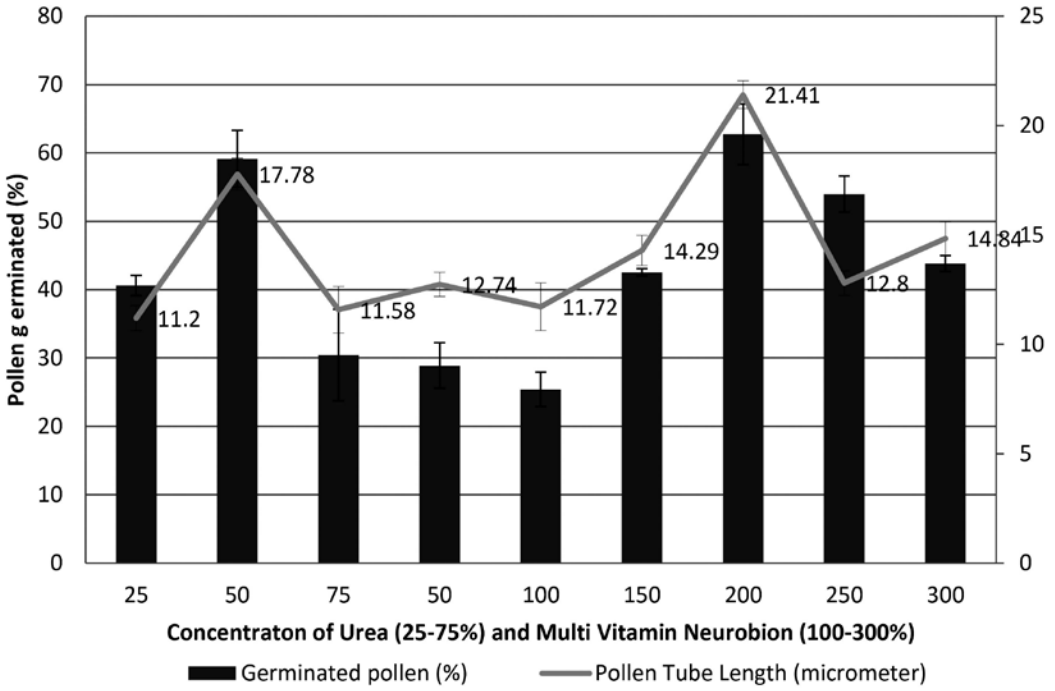

Fig. 4: Effect of Urea and Multi Vitamin Neurobion on Pollen Germination and Pollen tube length of neelakurinji *In vitro*

pollen grains which were intact and fluoresced strong yellow whereas non-viable grains were with faint color. (Fig. 6A&B). Generally sterile pollens were of 2-12% during the flowering period. Further pollen viability was tested by growing in artificial medium. Pollen viability at periodic interval revealed that fresh pollen showed 95.56% viability, the pollen viability was 78.74% after 48h(2nd day) and it has reached to 7.27% on 3rd day. Similarly, stigma receptivity was studied using Alexander stain which revealed stigma was receptive till 2nd day of anthesis *(*Fig.6C*)*

Scanning Electron Microscopy: The pollen morphology of both *S.kunthianus* and *S. wightianus (Fig.7)* are large, ellipsoidal in shape, aperture colpus, verrucate (*whereas it is perforate for S.wigtianus*) , monad, isopolar and bilateral in symmetry. The size of neelakurunji pollen was 26.44 x 64.25 µm and 30.14 x 81.12 µm for Kalkurunji. In kurunji pollen

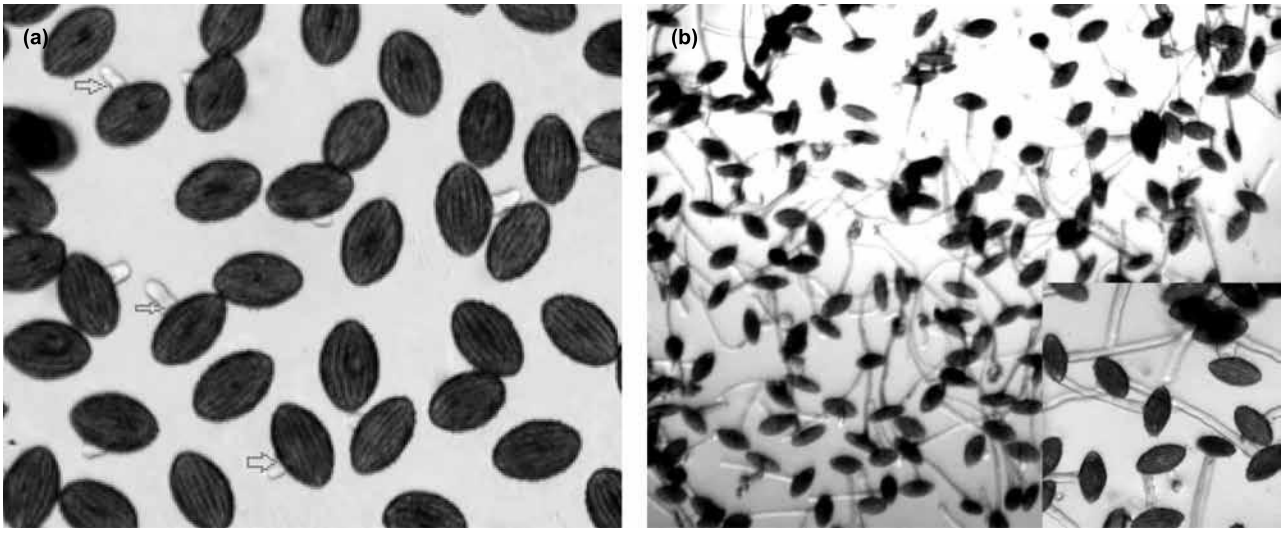

Fig. 5: Photomicrograph showing the Kurunji pollen (a) Pollens showing pollen tubes emerge from the belly of pollen (b) PGM showing pollen germination and pollen tubes

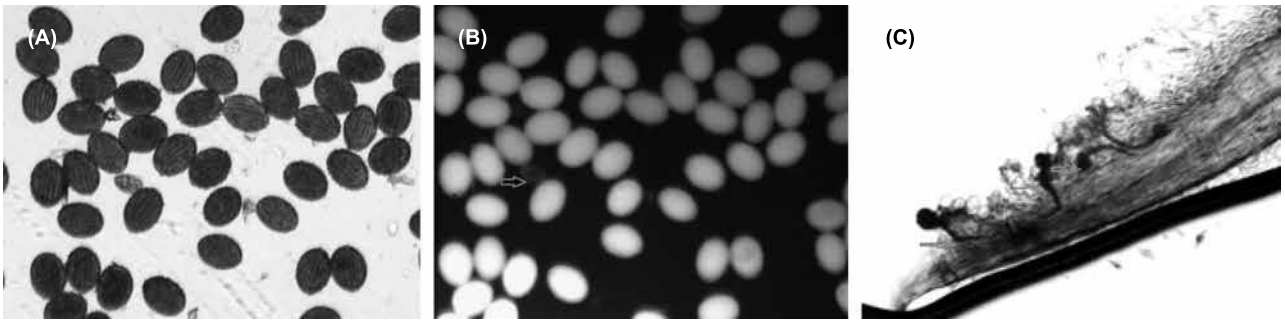

Fig. 6: Neelakurunji pollen : (a) Fresh pollen (b) FDA staining showing viable pollen stained darkly (*Arrows indicate sterile pollens*) (c) *Alexander staining indicates the pollen germination on stigmatic surface*

Fig. 7: Pollen morphology of Neelakurunji (a) Sample showing intact and sterile, x500 (b) Polar view showing germination pore in the middle x4000 (c) Neelakurunji Pollen with 26.74 × 60.16 µm in size, x4000 (d) Kalkurunji Pollen with 30.33 × 80.16 µm in size, x4000

strangly the germination pores were present in the middle (*Swollen region in middle*). Compared to Neelakurunji the size of kal kurunji pollen was larger.

The final composition of pollen medium for neelakurunji consist of sucrose 15% + PEG 4000 15% + 100 mgl^{-1} boric acid + 450 mgl^{-1} calcium nitrate +75 mgl^{-1} potassium nitrate+ 150 mgl^{-1} magnesium nitrate+ 50 mgl^{-1}urea+200 mgl^{-1}Neurobion forte(multivitamin tablet) Similarly, pollen germination medium for *Strobilanthus wigtianus*, another species commonly called as Kal kurunji has also been standardized. The PGM consists of *sucrose 15% + PEG 4000 15% + 100 mgl^{-1} boric acid + 300-350 mgl^{-1} calcium nitrate +100 mgl^{-1} potassium nitrate+ 200 mgl^{-1} magnesium nitrate+ agar* showed 60-75% pollen germination at 23°C after 45 minutes. Due to non-availability of pollen, the PGM could not be fine-tuned further.

AUTHORS' CONTRIBUTION

Conceptualization of research (P. Jayaprakash); Designing of the experiments (P. Jayaprakash and John Peter); Contribution of experimental materials (P. Jayaprakash and M. Sivasamy); Execution of field/ lab experiments and data collection (P. Jayaprakash John Peter and Rebekha Nisha); Analysis of data and interpretation (P. Jayaprakash and John Peter); Preparation of the manuscript (P. Jayaprakash, John Peter, Rebekha Nisha, Vikas, VK and M. Sivasamy).

DECLARATION

The authors declare no conflict of interest.

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