

The utility of electrospun nanofibre mats for in vitro germination of *Artabotrys hexapetalus* pollen

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Received 19 Mar 2015

Accepted 16 May 2016

ABSTRACT: This study investigates the influence of electrospun fibre mats on in vitro pollen germination of *Artabotrys hexapetalus* (L.f.) Bhandari (synonym: *A. odoratissimus* R. Brown). There was no difference in the size of the pollen from two closely related stages of flower development, namely the yellow florets and yellowish orange florets. On agar-based medium supplemented with 20% sucrose, about 50% of the pollen from the yellowish orange florets germinated but only 25% of those from the yellow florets did. More pollen from the yellowish orange florets germinated in liquid medium than on agar-gelled medium but pollen tube elongation was greater in agar-gelled medium. Electrospinning of synthetic polymer solutions of polyvinylidene fluoride (PVDF) or polylactic acid (PLA) was used to generate mats composed of nonwoven (randomly oriented) nanofibres as well as those composed of a mixture of nonwoven and aligned (PVDF4 and PLA4) nanofibres. Compared with liquid, agar medium, and other types of nanofibre mats, PVDF4 was found to be the best physical support substrate for germination of the pollen from the yellowish orange florets (70%) and tube growth (about 400% increase over that in liquid medium). Pollen germination was largely inhibited on all four types of 18 µm thick nanofibre mats but not on the 8 µm thick ones.

KEYWORDS: pollen viability, pollen tube growth, polylactic acid (PLA), polyvinylidene fluoride (PVDF)

INTRODUCTION

In vitro pollen germination tests are useful for assessing pollen viability and performance independent of the influence of the female reproductive system¹. The use of liquid or agar-gelled medium with the various ingredients is the most common physical support to germinate pollen in vitro. Earlier investigations have shown the importance of different ingredients including Ca²⁺ ions², boron³, and sucrose⁴ added to media for pollen germination of different plant species in vitro. A recent study shows that a solid support matrix such as a cellulose membrane placed on top of agarose gel resulted in a high percentage of *Arabidopsis* pollen germination in vitro⁵. Nevertheless, for each plant species it might be necessary to investigate the most appropriate method to get a high percentage of germination and good pollen tube growth.

Artabotrys hexapetalus (L.f.) Bhandari (Syn-

onym: *A. odoratissimus* R. Brown), an indigenous plant of Sri Lanka and the southern part of India, was introduced from China to Thailand for ornamental use. It bears profusely year-round extremely fragrant flowers which are sought after for perfume production, and is easy to propagate and maintain. Floral ontogeny, anther, and seed development of *A. hexapetalus* have been studied extensively^{6–9}, but the requirements for pollen germination in vitro of this Annonaceous plant have not been determined. This information might be helpful for assessing its reproductive system. In particular, the germination requirement of *A. hexapetalus* pollen was studied as a part of a larger study to build a better understanding as to why *A. hexapetalus* does not set fruit easily compared to other closely related species.

Electrospinning is a relatively simple technique to convert droplets of a polymer solution, under the influence of a high-voltage electrical field, into fibres of nanometres in diameter that can be collected in

Table 1 Electrospinning conditions for generation of PVDF and PLA nanofibre mats.

Polymer	Concentration (%, w/w)	Solvent	Voltage power supply (kV)	Travelling distance (cm)	Flow rate (ml/h)
PVDF	19	DMAC/acetone 4:6	10	10	1
PLA	12	THF/DMF 75:25	8	10	1

the form of a fibrous mat. Conventional nanofibre mats consist of fibres deposited in a random (nonwoven) pattern or in alignment with a particular predetermined pattern. It was shown that fibre alignment in the electrospun fibrous mats plays an important role on the microenvironment permissive for human nerve cell growth¹⁰. Recently, electrospun nanofibre mats consisting of a combination of both nonwoven fibres and those aligned to a specific pattern¹¹ were used as a novel physical support in a tissue culture medium for induction and growth of bilimbi callus¹². It was found that different types of electrospun nanofibre mats had different effects on the growth of bilimbi callus. There is also paucity of information on the possible influence of different physical supports for pollen germination in *A. hexapetalus*. The present work is aimed to investigate if electrospun nanofibre mats would be useful physical support matrices for germination of *A. hexapetalus* pollen.

MATERIALS AND METHODS

Pollen source

Pollen grains were collected from the dehiscent anthers of yellow and yellowish orange flowers of the *A. hexapetalus* plants grown in the campus of King Mongkut's University of Technology North Bangkok, Thailand. The pollen grains from flowers of the two developmental stages were collected separately and care was taken to ensure that there was no mixing of the pollen from the two stages of flower development during the experiment. A digital camera (Olympus C-760, Olympus Corporation) was mounted on a light microscope (ML2000, Meiji Techno Co., Ltd., Japan) for taking all the images of pollen germination and pollen tube lengths in this study.

Generation of nanofibre mats

Chemicals: polyvinylidene fluoride, Kynar 761 (PVDF) and polylactic acid, NatureWorks grade 4042D (PLA) were obtained from Benebiz Co., Ltd. (Bangkok) and NatureWorks LLC (Minnetonka, MN, USA), respectively. *N,N'*-dimethylacetamide (DMAC) and acetone were obtained from Fluka.

Tetrahydrofuran (THF) and *N,N'*-dimethylformamide (DMF) were obtained from Sigma Aldrich.

Electrospinning: PVDF and PLA nanofibre mats were electrospun using the concentrations of the respective polymer solutions under the processing conditions (Table 1).

Nonwoven nanofibre mats were electrospun on an aluminium (Al) sheet mounted on a rotating drum, and were referred to herewith as PVDF1 or PLA1 (Fig. 1). In addition, nanofibre mats composed of a mixture of nonwoven and aligned fibres were also produced under the same condition but first with an Al sheet, and then with a designed patterned plastic (Petty patent number 6223 issued by Department of Intellectual Property, Ministry of Commerce, Nonthaburi, Thailand) were used. The nanofibre mats produced in this way were referred herewith as PVDF4 or PLA4. At the completion of electrospinning, the nanofibre mats were released from the Al sheets and designed patterned plastics. Then the disks of nanofibre mats of either $8 \pm 2 \mu\text{m}$ or $18 \pm 2 \mu\text{m}$ thickness were punched using a paper hole puncher of 25 mm in diameter before they were laminated with plastic o-rings. The working surface area of the nanofibre mats was 21 mm in diameter. Samples of the nonwoven PVDF and PLA nanofibre mats were sent to the Scientific and Technological Research Equipment Centre, Chulalongkorn University for scanning electron microscope analysis.

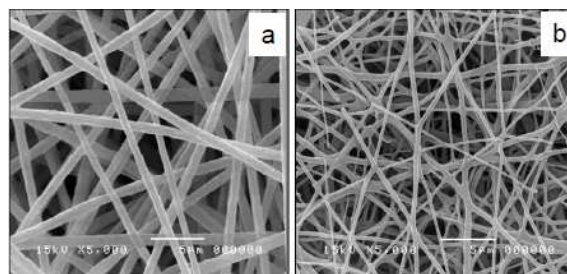


Fig. 1 Scanning electron microscope images of nonwoven nanofibre mats generated from electrospinning (a) polyvinylidene fluoride and (b) poly(lactic acid) polymer solutions. Magnification = 5000 \times .

Pollen germination tests

The basal liquid medium for in vitro pollen germination consisting of 0.1 mM H_2BO_3 and 1 mM $CaCl_2 \cdot 2H_2O$ was modified after that of Mercado et al.¹³. Unless specified otherwise, this basal medium was also supplemented with 20% (w/v) sucrose and then the pH of the medium was adjusted to 5.7 before sterilization in an autoclave at 121 °C and 15 psi for 20 min. After this, 10 ml of the liquid medium was poured into a sterile tissue culture dish (60 × 15 mm, Becton Dickinson Labware, NJ, USA). If required, agar (0.8%, w/v) was added to the basal medium before the sterilization step. Other pollen germination substrates investigated were the different electrospun nanofibre mats generated as described earlier here. A nanofibre mat was floated on the surface of the autoclaved basal medium by laying an edge of it down onto the surface of the medium first and then tenderly sliding the nanofibre mat down onto the surface of the medium.

Pollen grains were brushed off from the anthers of yellowish orange flowers and then dusted onto the surface of a germination support matrix (liquid, agar or a nanofibre mat). The dishes with pollen grains were moved carefully to a growth room for 24 h incubation at 30 ± 1 °C in the dark. For each of the six replications of a germination support matrix, ten fields under a light microscope were observed. Pollen grains were considered to have germinated when the emerged pollen tubes were longer than the diameter of the pollen¹⁴. Pollen tube length measurements were made after 24 h of germination with the aid of an ocular micrometre as viewed under a light microscope.

Statistical analysis

ANOVA of the data on germination percentages and pollen tube length was carried out. Based on the requirement, Duncan's MRT test was carried out at the significance level of $p < 0.05$.

RESULTS

Generally, the yellow petals of the *A. hexapetalus* flowers develop from the green bud stage in about 15 days and then they turn yellowish orange one day later as a normal course of floral development under natural growth conditions in Thailand. Under a light microscope, pollen grains from yellow and yellowish orange florets were of similar sizes after harvest and there was no change upon rehydration (unpublished observations).

The percentages of pollen germination in vitro

Table 2 In vitro germination tests using *A. hexapetalus* pollen from yellow and yellowish orange flowers on agar-gelled media supplemented with different concentrations of sucrose.

Sucrose (%)	Germination (%) [*]	
	Yellow flower	Yellowish orange flower
5	20.42 ± 0.63	27.96 ± 0.46
10	24.06 ± 0.46	34.80 ± 0.45
20	26.12 ± 0.41	50.35 ± 0.47

^{*} Data are mean ± standard error of 60 replications and all the values are significantly different (ANOVA, $p < 0.05$).

Table 3 In vitro germination tests using pollen from yellowish orange flowers in liquid medium or on different electrospun nanofibre mats floating on the surface of the liquid medium supplemented with 20% sucrose.

Support	Thickness of nanomats (µm)	Germination (%) [*]
Liquid medium	–	59.4 ± 1.3 ^d
PVDF1	8 ± 2	66.1 ± 1.2 ^{ef}
PVDF4	8 ± 2	70.22 ± 0.92 ^g
PLA1	8 ± 2	65.6 ± 1.3 ^e
PLA4	8 ± 2	68.5 ± 1.4 ^{fg}
PVDF1	18 ± 2	1.92 ± 0.53 ^a
PVDF4	18 ± 2	2.29 ± 0.45 ^a
PLA1	18 ± 2	0.30 ± 0.21 ^a
PLA4	18 ± 2	2.18 ± 0.52 ^a

^{*} Data are mean ± standard error of 60 replications and those sharing the same letter are not significantly different (ANOVA, $p < 0.05$).

from the yellow *A. hexapetalus* flowers only improved slightly (from 20–26%) with the increased concentrations of sucrose in the medium ranging from 5–20% (Table 2). In contrast, there was about two-fold increase in the percentage of pollen (50%) from yellowish orange flowers that germinated in the presence of 20% sucrose concentration as compared to that of 5% sucrose (Table 2). Based on these results, only pollen from the yellowish orange florets was used for further investigations.

Interestingly, the percentage of pollen (59%) from yellowish orange *A. hexapetalus* flowers germinated in liquid medium supplemented with 20% sucrose was significantly higher than those pollen grains that were germinated in agar-gelled medium with 50% sucrose of otherwise the same composition (Table 2 and Table 3; Fig. 2a and Fig. 2b). Another experiment was also carried out to investigate

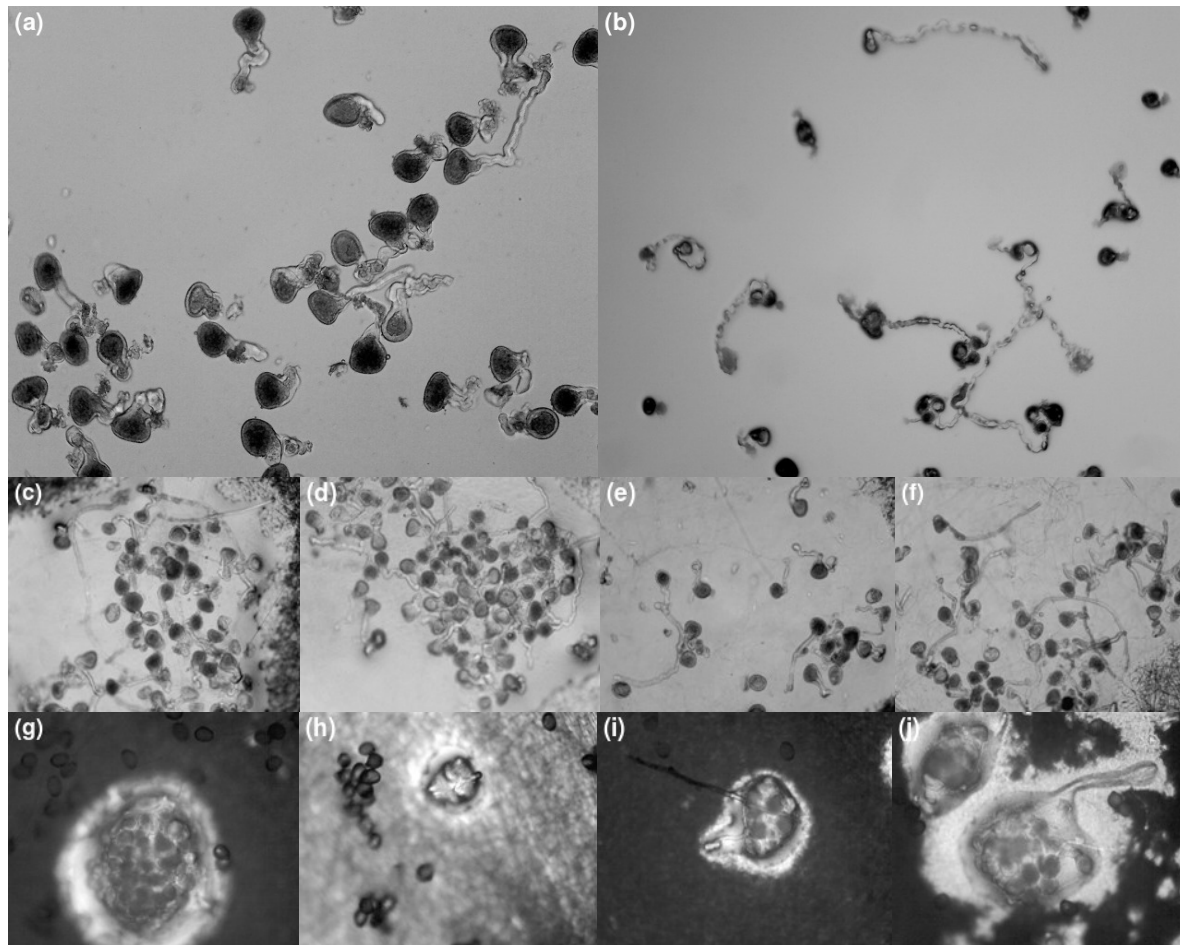


Fig. 2 Germination of *Artabotrys hexapetalus* pollen (a) in the basal liquid medium supplemented with 20% sucrose, (b) on the surface of agar-gelled basal liquid medium supplemented with 20% sucrose, and (c–j) on the surfaces of different electrospun nanofibre mats of either $8 \pm 2 \mu\text{m}$ or $18 \pm 2 \mu\text{m}$ floating on the basal liquid medium supplemented with 20% sucrose: (c) PVDF1, $8 \pm 2 \mu\text{m}$; (d) PVDF4, $8 \pm 2 \mu\text{m}$; (e) PLA1, $8 \pm 2 \mu\text{m}$; (f) PLA4, $8 \pm 2 \mu\text{m}$; (g) PVDF1, $18 \pm 2 \mu\text{m}$; (h) PVDF4, $18 \pm 2 \mu\text{m}$; (i) PLA1, $18 \pm 2 \mu\text{m}$; (j) PLA4, $18 \pm 2 \mu\text{m}$.

the germination of *A. hexapetalus* pollen on different types, patterns, and thicknesses of electrospun nanofibre mats floated on the liquid medium were initiated (Table 3; Fig. 2c–j). The best physical support for in vitro germination of *A. hexapetalus* pollen was the $8 \mu\text{m}$ thick PVDF4 or PLA4 nanofibre mats, resulting in about 70% germination (Table 3). The use of the nonwoven PVDF1 and PLA1 nanofibre mats, both $8 \mu\text{m}$ thick, resulted in slightly lower pollen germination (about 66%) than the patterned ones of the same thickness. Thicker ($18 \mu\text{m}$) electrospun nonwoven and patterned PVDF and PLA nanofibre mats were found to inhibit germination of *A. hexapetalus* pollen (Table 3).

Pollen tube length measurements revealed that pollen tube growth (length) on the surface of agar-

gelled medium ($392 \mu\text{m}$) was more than double compared to those that was grown in liquid medium ($142 \mu\text{m}$) (Table 4). This was contrary to the pollen germination results comparing liquid and agar medium (Table 2 and Table 3). Furthermore, it was found that PLA4 and PVDF4, both of $8 \mu\text{m}$ thick, were not only better for pollen germination but were also more beneficial for pollen tube growth (pollen tube length being about 30 and 40%, respectively, longer than those on agar-gelled medium).

DISCUSSION

In vitro germination of pollen has been extensively used for studying the genetical, physiological, biochemical and cytochemical characters for a wide range of plant species belonging to different fami-

Table 4 In vitro germination tests using pollen tube length after 24 h of germination on different germination supports.

Support	Pollen tube length (μm)
Agar medium	392 \pm 16
Liquid medium	142 \pm 15
PLA4 (8 \pm 2 μm thickness)	502 \pm 15
PVDF4 (8 \pm 2 μm thickness)	545 \pm 13

Data are mean \pm standard error of 30 replications and all the values are significantly different (ANOVA, $p < 0.05$).

lies. Pollen grains play a vital role in crop improvement programmes and have relative importance not only on fruit set but also on the flower-flower and flower-pollen interaction. The prime objective of the study was to investigate the possible innovative utility of electrospun nanofibre mats as a suitable physical support matrix for pollen germination in vitro of *A. hexapetalus*. The pollen germination medium was modified after that of Mercado et al¹³ and used without further optimization of the main chemical ingredients except sucrose concentrations.

In the present investigation, the type of *A. hexapetalus* pollen to be used was determined since pollen grains were produced by florets in two closely related flower developmental stages: yellow and yellowish orange florets. It is widely accepted that pollen from different plant species may require a specific amount of sucrose in an artificial medium for germination in vitro. This exogenous sucrose requirement has been found to play an important role as a nutritive resource and osmoregulator for pollen germination^{12, 15, 16}. The germination percentage of pollen from yellowish orange increased with the increasing concentrations of sucrose in the medium, suggesting that endogenous physiological differences of pollen might also play a role in regulation of pollen germination.

It has also been observed that the agar-based medium did not provide a sufficient physical support matrix for germination of *A. hexapetalus* on different types, patterns, and thickness of electrospun nanofibre mats floated on the liquid medium (Table 3; Fig. 2c–j). The results revealed that the appropriate electrospun nanofibres such as 8 μm thick PVDF4 was found to be a promising novel alternative germination substrates that could substantially improve *A. hexapetalus* pollen germination in liquid or agar-based medium. Under natural conditions, pollen grains germinate on the stigmatic surface which is a

biologically complex solid support matrix¹⁷. Liquid medium supports pollen hydration but it might not be the same as the fluid microenvironment on the surface of complex solid matrix. It is possible that certain nanofibre mats could better resemble the complex nature of that of the biologically complex stigmatic surface. The precise basis for the beneficial effects of the 8 μm thick PVDF4 for pollen germination and tube growth of *A. hexapetalus* over other types of physical supports, however, remains to be established.

Conclusively, electrospun nanofibre mats, particularly 8 μm thick PVDF4, influenced the in vitro pollen germination and tube growth of *A. hexapetalus*. Pollen quality is also better at yellowish orange coloured stage of petals in this plant. Further research needs to be carried out to optimize the medium composition and to see if these novel scaffolds could also be used to obtain better pollen germination and pollen tube growth in other plants besides *A. hexapetalus*.

Acknowledgements: We would like to thank Assoc. Prof. Boonyuen Kijwijan for his kind provision of sterile tissue culture dishes used in this work.

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