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Differential response of callus initiation and growth of aromatic chilli and bilimbi to two different electrospun nanofibre mats

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ABSTRACT: The development of new materials to be used as a substrate for plant tissue culture can lead to substantial advances in biotechnology. Here, mats consisting of a mixture of nonwoven (randomly oriented) and aligned nanofibres were produced by electrospinning solutions of polylactic acid (PLA) and polyvinylidene fluoride (PVDF). They were referred to as PLA4 and PVDF4, respectively. Callus initiation from stem explants of bilimbi (*Averrhoa bilimbi* L.) and aromatic chilli (*Capsicum frutescens* L.) occurred on these two types of nanofibre mats floated in liquid Murashige and Skoog (1962) basal medium supplemented with 2 mg/l α-naphthalene acetic acid or 2,4-dichlorophenoxy acetic acid, respectively. After subculturing for 3 weeks, the fresh weight of callus initiated from stem explants of bilimbi was significantly greater by 20% when PVDF4 rather than PLA4 nanofibre mats were used as a support matrix. In contrast, both the fresh and dry weights of callus initiated from stem explants of aromatic chilli were significantly greater by 11% and 50%, respectively, when PLA4 and not PVDF4 nanofibre mats was used. These differences in response to the two types of nanofibre mats were also found in relation to growth of callus of bilimbi and aromatic chilli during subculture. This is the first time electrospun nanofibre mats have been used in plant tissue culture research.

KEYWORDS: auxin, liquid medium, physical support matrix, polylactic acid (PLA), polyvinylidene fluoride (PVDF)

INTRODUCTION

Callus culture, a type of plant tissue culture consisting of undifferentiated tissues, constitutes an important tool in plant biotechnology. It can be used in numerous ways, for example, for organogenesis[1], indirect somatic embryogenesis[2], and generation of somaclonal variation[3].

Aromatic chilli (*Capsicum frutescens* L.) and bilimbi (*Averrhoa bilimbi* L.), two plants commonly used in Thai cuisine, were chosen for this study. There were prior studies on callus culture of the former[4] but there was no prior tissue culture work on the latter. Bilimbi is a small tree and has been classified as a member of the family Oxalidaceae. Although it is widely cultivated in the lowlands of Southeast Asia, it has been considered native to the tropical Americas[5]. Extracts of bilimbi leaves and fruits were also reported to have antibacterial activity[6].

Nanotechnology has the potential to make significant impacts in our society. For example, nanofibre, a threadlike structure on a nanometre scale, has new and useful properties for filtration, as protective materials, in electrical and optical applications[7]. Nanofibre mats have also been investigated as scaffolds or physical support matrices for various types of tissue regeneration including skin, blood vessel, cartilage, bone, and nerve[8–10]. However, there has been no application of nanofibre mats in plant science and biotechnology. Delivery systems for nanoparticles into plants have been investigated[11,12] but generally there is a paucity of applications of nanotechnology in plant science and biotechnology.

Electrospinning is a relatively simple technique to convert droplets of a polymer solution, under the influence of a high-voltage electrical field, into nanofibres forming fibrous mats for various applications[13,14]. The orientation or arrangement of nanofi-

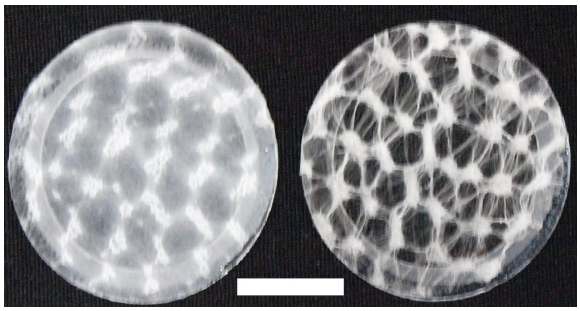

Fig. 1 PLA4 (left) and PVDF4 (right) nanofibre mats used in this experiment (each one was 2.54 cm in diameter). The nanofibre mats in the images consisted of a mixture of aligned and nonwoven nanofibre. The dots on the images are parts of nonwoven nanofibre interconnected with aligned nanofibre.

bres in a nanofibre mat can be manipulated to be random (nonwoven), or aligned in some predetermined design, or a mixture of both[15]. Polylactic acid (PLA) and polyvinylidene fluoride (PVDF) are two of the many polymer solutions that have been electrospun into fibrous mats for investigations into their potential practical applications. PLA is a biocompatible, biodegradable, and environmentally friendly composite that has the potential for environmental and biomedical applications[16]. PVDF is extensively used for filtration membranes and in rechargeable batteries due to its outstanding chemical resistance and good thermal stability[17].

Nanofibre mats might be of use in plant tissue culture but it is obvious that they cannot be considered to be an alternative to agar or other support matrices for micropropagation[18], primarily from the cost perspective alone. However, different nanofibre mats, being novel artificial materials, could unexpectedly result in different in vitro plant cell growth responses. The objective of the present study was to investigate this possibility by comparing the utility of two different types of nanofibre mats, referred herein as PLA4 and PVDF4 (Fig. 1), for callus initiation and growth during subculture of aromatic chilli and bilimbi.

MATERIALS AND METHODS

Nanofibre preparation

(a) Chemicals: PVDF (Kynar 761) with an MW of 441 000 was supplied by Benebiz Co., Ltd. (Bangkok). Acetone, chloroform ($CHCl_3$), dimethylformide (DMF), and N,N-dimethylacetamide (DMAc) were purchased from Acros Co., Ltd. (Hull, UK). PLA (NatureWorks grade 4042D) was kindly supplied by NatureWorks LLC (Minnetonka, USA).

(b) Preparation of polymer solutions: PLA solution (8%, w/v) was prepared in $CHCl_3$/DMF (75/25, v/v), whereas PVDF solution (19%, w/v) was prepared in acetone/DMAc (6/4, w/w).

(c) Electrospinning: The electrospinning setup consisted of a 5-ml plastic syringe containing an appropriate polymer solution that was connected to a steel needle (0.41 mm diameter) as an injection system moving horizontally at a speed of 6 cm/s. The needle was connected to a high voltage power supply. Nonwoven nanofibre were first electrospun onto an aluminium (Al) sheet (as a backing substrate) mounted on a rotating drum (15 cm diameter rotating at a speed of 150 r.p.m.). After this, the Al sheet was replaced with a plastic sheet of a desired pattern (Petty patent number 6223 issued by Department of Intellectual Property, Ministry of Commerce, Nonthaburi, Thailand). Electrospinning was resumed to generate a mat with a mixture of nonwoven and aligned nanofibre (Fig. 1). The plastic sheet was used as backing substrate and not as part of the nanofibre mats. The PVDF nanofibres exhibited a diameter distribution of 400–1000 nm while the PLA nanofibres were 200–700 nm in diameter.

Electrospinning was performed at a voltage of 10 kV, a distance (referred to as the travelling distance) between the needle tip and the grounded collector of 10 cm, and a flow rate of 1 ml/h. All the electrospinning manipulations were carried out at 25–28 °C and 70–75% humidity. The thickness of the nanofibre mat used in this study was about 15 μm.

Plant materials and culture conditions

Plant materials and surface-sterilization: Ripe bilimbi (*A. bilimbi*) fruits were collected from an orchard in Muang district, Trat province, Thailand. Seeds were isolated from the fruits, washed with tap water, and surface sterilized for 15 min with 15% (v/v) Clorox (a commercial bleach solution containing 5.25%, w/w, sodium hypochlorite as available chlorine) to which 2–3 drops of Tween 20 were added. Then, they were rinsed 3 times (1 min each time) with sterile distilled water. After the arils that were still attached to the surface-sterilized seeds had been removed, the seeds were immersed in sterile distilled water again for 1 min in 10% (v/v) Clorox for 10 min and rinsed 3 times (1 min each time) with sterile distilled water.

Seeds of aromatic chilli (*C. frutescens*) were purchased from Thai Seed and Agriculture Co., Ltd., Bangkok. Surface-sterilization began with immersing the seeds in distilled water overnight before soaking for 15 min in 15% (v/v) Clorox to which 2–3 drops of Tween 20 were added. After this, they were rinsed

3 times with sterile distilled water (1 min for each rinse).

Germination of seeds and explant preparation

Surface-sterilized bilimbi and aromatic chilli seeds were placed onto basal MS medium[19] without any plant growth regulators, gelled with 0.8% agar (w/v), and kept under 16 h of illumination with white fluorescent lamps (average light intensity of 47.31 µmol m^{-2} s^{-1} at the top of the culture jars) and 8 h of darkness in a growth room at 25 ± 2 °C. Stem explants (1 cm long) were excised from below the last node of 8-week-old bilimbi plants (approximately 5–5.5 cm tall) or 4-week-old seedlings (approximately 4–4.5 cm tall) of aromatic chilli grown from the respective surface-sterilized seeds.

Callus culture

Initiation: For callus initiation, a stem explant of bilimbi or aromatic chilli was placed horizontally for 3 weeks on the surface of: (a) a PLA and (b) a PVDF nanofibre disc, each of 2.54 cm cut out from a nanofibre mat using a cork borer. The medium used in both treatments for bilimbi or aromatic chilli was liquid basal MS medium (3 ml) containing 2 mg/l α-naphthalene acetic acid (NAA) or 2,4-dichlorophenoxy acetic acid (2,4-D), respectively.

Subculture: For the callus subculture experiment, stem explants of bilimbi or aromatic chilli were first cultured for 3 weeks on basal MS medium supplemented with 2 mg/l NAA or 2,4-D, respectively, gelled with 0.8% (w/v) agar (Fig. 2). The callus thus initiated was used for subculture in the same 2 aforementioned treatments (a) and (b) for 3 weeks. A piece of subcultured callus (0.8–1 cm width × 0.8–1 cm length) was placed on an appropriate nanofibre mat.

All media in this study were adjusted to pH 5.7 and placed in glass containers (8.5 cm tall × 4.5 cm diameter) together with the appropriate nanofibre mat before they were autoclaved at 121 °C and 15 psi for 20 min.

Determination of fresh and dry weights

At the end of every week during callus induction and subculture, bilimbi or aromatic chilli stem explant+callus formed on a nanofibre mat was weighed and then reweighed after drying for 6 h in an oven at 105 °C. The difference between fresh and dry weight at a given callus harvest time was taken as the water content of the calli at that time.

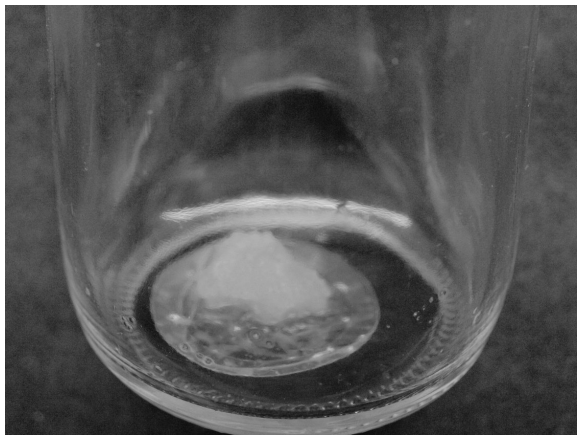

Fig. 2 Initiation of callus from stem explants cultured on a PLA4 nanofibre mat for 3 weeks.

Data analysis

There were six replicates for each treatment at a particular data collection time. Mean percentages of fresh and dry weight ± SD were analysed and one-way ANOVA was first performed at the significance level of $P < 0.05$. After this, when appropriate, Duncan comparison of means was carried out at $P < 0.05$.

RESULTS AND DISCUSSION

The protocol for callus initiation and subculture of bilimbi was developed following preliminary experiments. That for aromatic chilli was similar to a previously published protocol[20], except that stem explants were isolated from 4-week-old seedlings and kinetin was not used. In preliminary experiments, it was found that there was little difference in initiation and growth of callus of stem explants of bilimbi and aromatic chilli when subculturing on agar-solidified MS medium supplemented with 2 mg/l NAA or 2 mg/l 2,4-D, respectively (data not shown). The main aim of the present study was to investigate the possibility that different nanofibre mats might have some properties/structure that could generate different physical/surface phenomena for different in vitro plant growth responses.

Three ml of plant tissue culture medium were found to be sufficient to support callus growth for 3 weeks. The nanofibre mats remained afloat in this volume of medium without submerging parts of the cultured materials that might have undesirable physiological effects on the cultured materials. It would seem worthwhile in future studies to include experimental determination of the thickness of a nanofibre

Table 1 Changes of fresh and dry weights of calli induced from bilimbi stem explants during three weeks of culture using two different types of nanofibre mats on liquid basal MS medium supplemented with 2 mg/l NAA.

Mat	Week 0	Week 1	Week 2	Week 3
(A) Fresh weight, average ± S.E. (mg)				
PLA4	4.8 ± 0.2^a	46.7 ± 1.3^a	154 ± 13^a	319 ± 3^a
PVDF4	4.7 ± 0.3^a	56.3 ± 1.9^b	182 ± 10^b	383 ± 13^b
(B) Dry weight, average ± S.E. (mg)				
PLA4	1.3 ± 0.1^a	5.0 ± 0.3^a	8.3 ± 0.3^a	12.3 ± 0.6^a
PVDF4	1.3 ± 0.1^a	4.7 ± 0.6^a	10.9 ± 0.6^a	14.4 ± 0.4^a

Means within a column in (A) or (B) assigned with a different letter were significantly different.

Table 2 Changes of fresh and dry weights of calli induced from aromatic chilli stem explants during three weeks of culture using two different types of nanofibre mats on liquid basal MS medium supplemented with 2 mg/l 2,4-D.

Mat	Week 0	Week 1	Week 2	Week 3
(A) Fresh weight, average ± S.E. (mg)				
PLA4	4.3 ± 0.2^a	16.3 ± 0.5^b	69 ± 4^b	110 ± 4^b
PVDF4	4.3 ± 0.2^a	11.6 ± 0.4^a	59 ± 5^a	99 ± 6^a
(B) Dry weight, average ± S.E. (mg)				
PLA4	1.3 ± 0.1^a	3.4 ± 0.2^b	7.4 ± 0.4^b	10.0 ± 0.4^b
PVDF4	1.3 ± 0.1^a	3.2 ± 0.2^a	5.7 ± 0.7^a	6.7 ± 0.3^a

Means within a column in (A) or (B) assigned with a different letter were significantly different.

mat and volume of medium required to achieve an optimal/desirable experimental outcome of plant tissue culture.

Both types of nanofibre mats used in the present study supported initiation and growth of calli in stem explants of bilimbi and aromatic chilli. There was a continuous increase in fresh and/or dry weights of the calli of bilimbi and aromatic chilli on both types of nanofibre mats throughout the experiment (Table 1 and Table 2). From the first week of callus induction in bilimbi stem explants, the fresh weight of the cultured material on PVDF4 nanofibre mats was already higher than that on PLA4 nanofibre mats. This difference was also evident in weeks 2 and 3 (Table 1). Thus, at the end of the experiment, calli initiated from bilimbi stem explants had a higher fresh weight (by 20%) when PVDF4 rather than PLA4 nanofibre mats were used under an otherwise comparable culture environment and time frame. In contrast, there was no difference in the dry weights of the calli over 3 weeks on both types of nanofibre mats indicating that throughout this experiment there was a greater increase in the water contents of the bilimbi calli initiated on PVDF4 nanofibre mats.

Interestingly, calli initiated from aromatic chilli stem explants on PLA4 nanofibre mats had both higher fresh and dry weights than those cultured on PVDF4 nanofibre mats throughout the experiment (Table 2). Thus, at the end of week 3, calli cultured on PLA4 nanofibre mats had higher fresh and dry weights by 11% and 49%, respectively, indicating that more biomass was accumulated when the calli of aromatic chilli were initiated on PLA4 nanofibre mats.

The fresh and dry weights of the bilimbi and aromatic chilli calli increased throughout 3 weeks of callus subculture (Table 3 and Table 4). Therefore, difference in callus initiation of the two different

Table 3 Comparison of growth of subcultured calli of bilimbi on two different types of nanofibre mats. Changes of fresh and dry weights of callus during three weeks of culture on liquid basal MS medium supplemented with 2 mg/l NAA.

Mat	Week 0	Week 1	Week 2	Week 3
(A) Fresh weight, average ± S.E. (mg)				
PLA4	121 ± 2^a	169 ± 4^a	584 ± 24^a	1080 ± 40^a
PVDF4	120 ± 5^a	179 ± 8^b	790 ± 23^b	1220 ± 40^b
(B) Dry weight, average ± S.E. (mg)				
PLA4	4.5 ± 2.4^a	13 ± 7^a	33.4 ± 3.0^a	42.8 ± 2.9^a
PVDF4	4.5 ± 2.6^a	13.8 ± 1.0^b	37.7 ± 3.2^b	49.0 ± 2.8^b

Means within a column in (A) or (B) assigned with a different letter were significantly different.

plants dependent on the type of nanofibre mat used was similarly observed during callus subculture. This might be related to the different properties of the nanofibre mats used. For example, PVDF4 nanofibres are nonpolar whereas PLA4 nanofibres are polar. In preliminary observations, it was estimated that PVDF4 nanofibre mats had a higher porosity than PLA4 nanofibre mats. Possibly, callus initiation and subculture in bilimbi stem explants had preference on less polar and more porous substrate (such as PVDF4 nanofibre mats). Callus initiation and subculture in aromatic chilli might have the opposite preference (hence PLA4 nanofibre mats). Further studies, however, are needed to elucidate this and other relationships between properties/structures of nanofibre mats and responses of plant tissue culture.

In conclusion, these results suggest that electrospun nanofibre mats might be more than just as inert physical support matrices for plant tissue culture. It seems worthy of future studies to investigate in depth the utility of different types of nanofibre mats for

Table 4 Comparison of growth of subcultured calli of aromatic chilli on two different types of nanofibre mats. Changes of fresh and dry weights of callus during three weeks of culture on liquid basal MS medium supplemented with 2 mg/l 2,4-D.

Mat	Week 0	Week 1	Week 2	Week 3
(A) Fresh weight, average ± S.E. (mg)				
PLA4	123 ± 3^{a}	222 ± 5^{b}	376 ± 9^{b}	1220 ± 12^{b}
PVDF4	123 ± 4^{a}	172 ± 4^{a}	319 ± 7^{a}	1165 ± 16^{a}
(B) Dry weight, average ± S.E. (mg)				
PLA4	5.3 ± 0.2^{a}	23.0 ± 0.5^{b}	27.4 ± 0.6^{b}	59.2 ± 0.6^{b}
PVDF4	5.3 ± 0.2^{a}	19.5 ± 0.9^{a}	24.4 ± 0.3^{a}	39.9 ± 0.8^{a}

Means within a column in (A) or (B) assigned with a different letter were significantly different.

different plant tissue culture systems and processes such as in vitro production of secondary metabolites[21] and somatic embryogenesis[2]. In particular, research currently underway includes utility of nanofibre mats manufactured with different materials possessing different chemical and physical properties that will allow comparison of their effects on plant growth and development.

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