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Adventitious Root Cultures of *Boesenbergia rotunda* as a Source of Pinostrobin

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***Abstract*—Pinostrobin is the major bioactive compound from the roots of *Boesenbergia rotunda,* and it possesses activities against HIV, *Helicobacter pylori,* ulcer, leukemia and inflammatory. The present study aimed to optimize the conditions for the production of adventitious roots from *B. rotunda* on Murashige and Skoog (MS) solid medium. The effects of the MS strength and sucrose concentration on adventitious root growth were studied as well as the production of pinostrobin. Adventitious roots were initiated from bud explant of *B. rotunda,* on MS solid medium supplemented with 2.0 mg/L 1-Naphthaleneacetic acid (NAA) and were used to establish root suspension cultures. The optimum biomasses of 1.83 ± 0.21 g fresh weight were obtained with a 1/2 strength of MS medium. High pinostrobin production (3.54 mg/g) was obtained with 50 g/L of sucrose concentration. These results suggest that adventitious root cultures are suitable for the raw material for the large-scale production, which contain a high yield of the pinostrobin compound. The results also revealed that the culture condition is a good strategy for enhancing the pinostrobin content in the adventitious root cultures.**

***Keywords*— adventitious root; *Boesenbergia rotunda*; Zingiberaceae; culture condition; pinostrobin**

I. INTRODUCTION

Plants with medicinal values have long been used in the development of human culture to treat various of illnesses. Medicinal plants or herbs are the main resources of traditional medicines. These plants also acted as an initial model to synthesize many of the modern drugs nowadays [1]. *Boesenbergia rotunda* L. which was formerly known as Boesenbergia or *Kaempferia pandurata* (Roxb. Schltr.) is a small perennial monocotyledonous plant belongs to the Zingiberaceae family. This plant is locally known as 'Temu kunci' in Malaysia and Indonesia. It is believed to be originated from India, South-East Asian and South China. *B. rotunda* is well known by its pharmacological worth through the presence of important flavonoids, essential oils, and chalcones. The rhizomes of this plant have always been traditionally used as a remedy for peptic ulcer, as well as colic, oral diseases, urinary disorders, dysentery, inflammation [2], gastrointestinal disorder, and aphrodisiac [3]. *B. rotunda* requires a long period of time to produce in large scale as this plant is conventionally propagated through vegetative technique via rhizome segments. Furthermore, this underground plant is highly prone to soft rot and rhizome diseases, which easily transmitted through traditional practices. It is also proved that the infection effects on younger stage plants will be more severe and leads to unexpected mass scale destruction [4]. Application of tissue culture techniques is considered a solution to this problem [5].

In vitro techniques is known to be more consistent, can be controlled and relatively more stable. Shoot cultures and root cultures for the production of medicinally important compounds have been attempted, but no scientific report on induction of adventitious root in *B. rotunda*. Based on previous studies, organ cultures produce higher and more stable secondary metabolites in differentiated tissues [6]. It has been reported that the highest bioactive compound in *B. rotunda* is pinostrobin, which possessed many

pharmacological usages [7], such as anti-Helicobacter pylori activity [8], an anti-leukemia activity [9], anti-HIV activity [7] anti-aromatase activity [10], reduce estrogen-induced cell proliferation activity [11] and antimutagenic activity [2].

In nature, production of secondary metabolites exists in small amounts [8]. The production of secondary metabolites by conventional method has numerous weaknesses such as low yields and fluctuates in concentrations due to geographical, seasonal, and environmental variations [12]. The adventitious root culture is considered as an effective technique to produce constant biomass and secondary metabolites [13], [14]. This scientific investigation was carried out with the aim to establish a protocol to enhance the biomass of adventitious roots as well as the pinostrobin content.

II. Material and Methods

A. Effects of Culture Conditions on Root Biomass

The well established *in vitro* adventitious roots that were cultured onto MS medium supplemented with 2.0 mg/L NAA [10] was used as the initial material. The adventitious roots were transferred onto MS medium with different MS strengths (¼, ½, ¾, 1, 1½ and 2) and sucrose concentrations (0%, 1%, 3%, 5%, 7% and 9%); each was supplemented with 2.5 g/L gelrite and 2.0 mg/L NAA. The pH of all media was adjusted to 5.75 to 5.80 with 1.0 M sodium hydroxide (NaOH) and/or 1.0 M hydrochloric acid (HCl) prior to autoclaving. The cultures were maintained in a growth room at 25 ± 2ºC under dark condition. Fresh weight (FW), number of roots and length of roots were recorded after eight weeks of culture.

B. Experimental Design and Statistical Analysis

The data of each parameter were analyzed using One-Way ANOVA followed by mean comparison with Tukey B test at $p \leq 0.05$ using SPSS.

C. Quantification of Pinostrobin Content from Adventitious Root Cultures

1) Plant Extraction: The adventitious roots were cleaned, sliced, oven dried at 38 °C, ground into powder and soaked in methanol. The samples were filtered, and the crude extract was partitioned against an equal volume of ethyl acetate and water added with sodium chloride (NaCl). The procedures were repeated three times before added sodium sulphate (Na_2SO_4) anhydrous into the crude extract and remained overnight. The samples were then again evaporated. The mass of the partitioned ethyl acetate extract was recorded and re-dissolved in methanol HPLC grade at a ratio of 1 mg of extract to 100 µl methanol HPLC grade. This methanolic solution of the extract was filtered through 0.45 µm PTFE filter (Sartorius 13 CR) prior to HPLC injection.

2) HPLC Analysis: The injection volume was adjusted to 20 µl per injection and was observed at 285 mm to 330 mm of wavelength in an Agilent 1200 Series HPLC system consisted of a G1323B quaternary pump with solvent cabinet, automatic injector (AI), G1315B diode array detector (DAD) with standard flow cell (10 mm path length, 13 µl volumes, 400 bar maximum pressure). The reverse column used was a Zorbax 300SB-C18C (150 x 4.6 mm i.d.; 5 µm). The solvent system used was 80% phosphoric acid and 20% acetonitrile for 0.5 min. This was subsequently mixed using a linear gradient starting with 80% water (containing 0.1% phosphoric acid) decreasing (over 5 min) then to 65% (over 5 min) to 40% (over 5 min), and finally to 0% (over 8 min). All compounds were detected and quantified by matching their retention times and spectral characteristics with known standards that had been identified previously [24].

III. Results and Discussions

A. Effect of Culture Conditions on Adventitious Root Growth and Pinostrobin Content of B. Rotunda

The effect of MS strength and sucrose concentration were evaluated in term of fresh weight, number of adventitious roots and length of adventitious roots produced for each treatment as shown in Table 1. In terms of nutrients requirement, different plants entail varying content of nutrients for growth [15]. Based on Table 1, data recorded after eight weeks of culture revealed that between all MS medium strengths, the fresh weight of adventitious roots cultured on the ½ strength of MS medium was found to produce the highest root biomasses with a mean value of 1.83 ± 0.21g FW. The mean value of fresh weight obtained from adventitious roots cultured on 1/4 MS strength was 1.20 ± 0.21, followed by 3/4, 1, 1½ and 2 MS strength with mean values of 0.78 ± 0.25, 0.77 ± 0.23, 0.61 ± 0.26 and 0.60 ± 0.34 g FW respectively.

The number of adventitious roots of *B. rotunda* cultured on 1/4, 1/2 and 2 MS strength medium was significantly different, whereas the number of adventitious roots cultured on 3/4, 1 and 1½ MS strength medium was found not significantly different. The highest number of adventitious root was obtained when cultured on 1/2 MS strength medium with a mean value of 13.13 ± 1.98, with numerous formation of secondary roots. This result was then followed by the adventitious roots produced on 1/4, 3/4, 1 and 1 ½ MS strength medium with a mean value of 10.13 ± 0.59, 6.88 ± 1.87, 5.00 ± 1.41 and 4.88 ± 1.10 respectively where all main roots are forming secondary roots. The lowest number of adventitious roots was obtained when cultured on 2 MS strength medium with a mean value of 2.50 ± 0.76 and did not produce any secondary root.

In addition, the length of adventitious roots of *B. rotunda* obtained when cultured on 3/4, 1/2 and 2 MS strengths in the medium were found to be significantly different, whereas the adventitious roots cultured on 3/4, 1 and 1½ MS strength were not significantly different. The highest length of adventitious roots was obtained when cultured on 1/2 MS medium with a mean value of 21.69 ± 1.69cm and many secondary roots formed, whereas the length of adventitious roots cultured on 2 MS strength medium was found to be the lowest with a mean value of 4.21 ± 1.07 cm and did not produce any secondary roots. The mean value of the length of adventitious roots obtained from adventitious roots cultured on 1/4 MS strength medium was 15.03 ± 1.64 cm, followed by 3/4, 1 and 1½ MS strength medium with a mean value of 10.10 ± 2.12, 8.36 ± 2.21 and 6.34 ± 1.21cm respectively where all produced secondary roots. This result

also links hand in hand with the morphological observations showed in Fig. 1.

This showed correlation to the other previous reports on the role of medium strength to enhance the adventitious root biomass in cell, tissue and organ cultures of several plant species. For instance, the ½ strength of MS medium was proven to significantly increase the biomass and phenolic compound in adventitious roots of *Echinacea angustifolia* [16]. Meanwhile [17] stated that both ½ and full MS strength medium were capable of enhancing the biomass of adventitious roots whereas full MS strength medium was found significant to elevate the secondary metabolite accumulation in adventitious roots of *Panax ginseng*. From this investigation too, we discovered that regardless of the increased in MS strength (1, 1 ½ and 2) the adventitious root biomass together with number and length of adventitious roots of *B. rotunda* showed a significant reduction (Table 1). The highest pinostrobin content (3.43 ± 0.01 µg/g) also was attained in adventitious roots that cultured on ½ MS strength medium (Table 2). These results indicated that the culture of adventitious roots of *B. rotunda* required an only low level of MS strength to produce high root biomass and pinostrobin content. The results of adventitious roots growth which include fresh weight, number of adventitious roots and root length were correlated with the quantification analysis of pinostrobin contents from all the MS medium salt strengths treatments. Half (1/2) MS was found to have the capability to produce highest pinostrobin content with the concentration of (3.43 ± 0.01 µg/g) (Table 2). From the table also showed that as the MS strengths increased the pinostrobin contents decreased. Detection of pinostrobin using HPLC through the appearances of chromatogram peaks in Fig. 2 also displayed a correlation with the results in (Table 2).

TABLE I
THE FRESH WEIGHT, NUMBER OF ADVENTITIOUS ROOTS AND LENGTH OF ADVENTITIOUS ROOTS PRODUCED FROM EACH TREATMENT WITH DIFFERENT MS STRENGTH AND SUCROSE CONCENTRATION AFTER EIGHT WEEKS IN CULTURES

Culture condition	Treatment	Fresh weight (FW) (g)	Number of adventitious roots	Length of adventitious roots (cm)
MS strength	¼	1.20 ± 0.21 ac	10.13 ± 0.59 a	15.03 ± 1.64 a
	½	1.83 ± 0.21 b	13.13 ± 1.98 b	21.69 ± 1.69 b
	¾	0.78 ± 0.25 ac	6.88 ± 1.87 c	10.10 ± 2.12 c
	1	0.77 ± 0.23 ac	5.00 ± 1.41 c	8.36 ± 2.21 c
	1 ½	0.61 ± 0.26 acd	4.88 ± 1.10 c	6.34 ± 1.21 c
	2	0.60 ± 0.34 acd	2.50 ± 0.76 d	4.21 ± 1.07 d
Sucrose concentration	0%	N.A	N.A	N.A
	1%	0.56 ±0.16 a	3.38 ±1.13 a	3.83 ±1.31 a
	3%	0.78 ±0.25 a	5.00 ±1.41 a	8.36 ±2.21 b
	5%	2.36 ±0.51 b	9.12 ±3.06 b	19.94 ± 2.49 c
	7%	0.70 ±0.41 a	6.25 ±1.50 ab	7.74 ±1.88 b
	9%	0.74 ±0.38 a	3.75 ±0.50 a	4.59 ±0.97 c

Values represent mean ± SE for 8 replicates

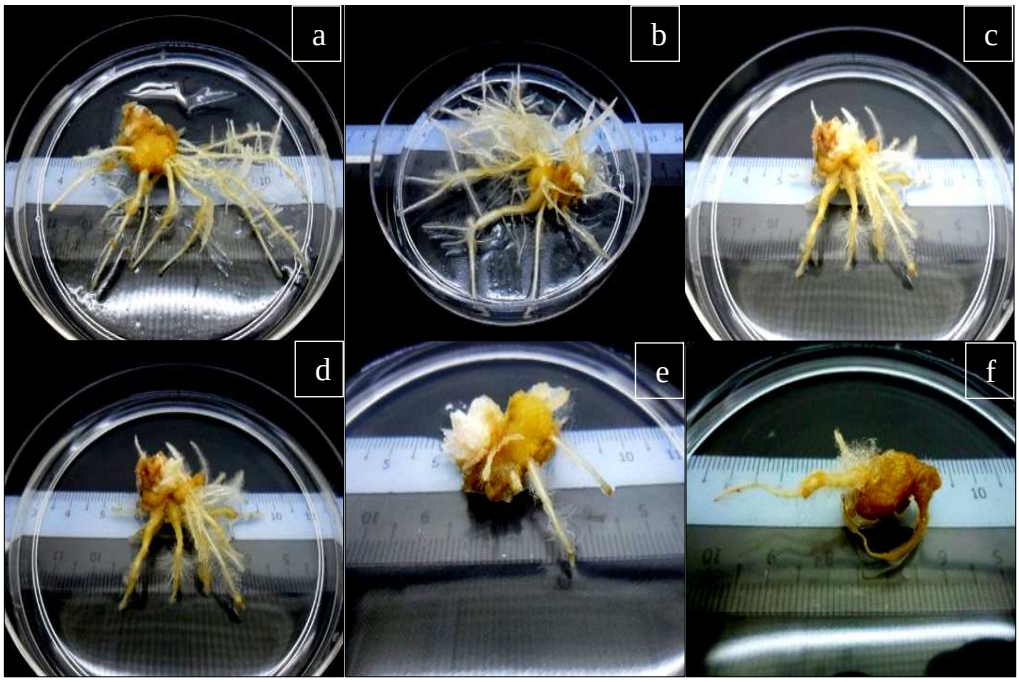

Fig. 1 Adventitious roots produced in different strength of MS medium; (a) ¼ ; (b) ½ ; (c) ¾ ; (d) 1 ; (e) 1 ½ ; (f) 2

However, the number and length of adventitious roots vary for (3, 7 and 9)% (w/v) with mean value of 5.00 ± 1.41, 8.36 ± 2.21 cm; 6.25 ± 1.50, 7.74 ± 1.88 cm; and 3.75 ± 0.50, 4.59 ± 0.97 cm respectively. The higher the sucrose concentration, the lower the number and length of adventitious roots produced. The adventitious root biomass cultured in MS medium supplemented with 1% (w/v) sucrose produced the lowest quantities of fresh weight 0.56 ± 0.16 g FW as well as the number and length of adventitious root with mean values of 3.38 ± 1.13 and 3.83 ± 1.31 cm respectively. This may due to not sufficient energy supplied. The relatively high concentration of sucrose in the present study was noticeably suitable for adventitious root growth in terms of its FW, number, and length of adventitious roots, here as too low or too high sucrose concentration was not appropriate as it may supply not enough energy for root initiation as well as reduced adventitious root growth characteristics respectively.

Meanwhile, the root growth (biomass, number, and length of adventitious roots) showed a significant reduction in MS medium supplemented with 9% (w/v) sucrose. This may be due to higher osmotic pressure in the medium with the rising of sucrose concentration that gave deleterious effects to the root induction [18], [19], [20]. The adventitious root biomass cultured in MS medium supplemented with 1% (w/v) sucrose produced the lowest adventitious root biomass together with the number and length of adventitious roots (Table 1). These could be observed through obvious adventitious root growth in Fig. 4. Therefore, it could be specified that reasonably low concentration of sucrose was suitable for adventitious roots growth whereas too low or too high sucrose concentration was not appropriate as it might not supply sufficient energy for root initiation which soon reduces the adventitious roots growth characteristics respectively.

TABLE II
THE CONCENTRATION OF PINOSTROBIN OBTAINED FROM EACH TREATMENT WITH DIFFERENT MS STRENGTH AND SUCROSE CONCENTRATION

Culture condition	Treatment	Concentration of Pinostrobin (µg/g)
MS strength	¼	1.15 ± 0.01 a
	½	3.43 ± 0.01 b
	¾	0.51 ± 0.01 a
	1	0.13 ± 0.00 a
	1 ½	0.08 ± 0.01 a
	2	0.03 ± 0.00 a
Sucrose concentration	0%	N.A
	1%	0.05 ± 0.00 a
	3%	0.13 ± 0.00 a
	5%	3.61 ± 0.02 b
	7%	2.15 ± 0.01 b
	9%	0.15 ± 0.00 a

Values represent mean ± SE for 3 replicates

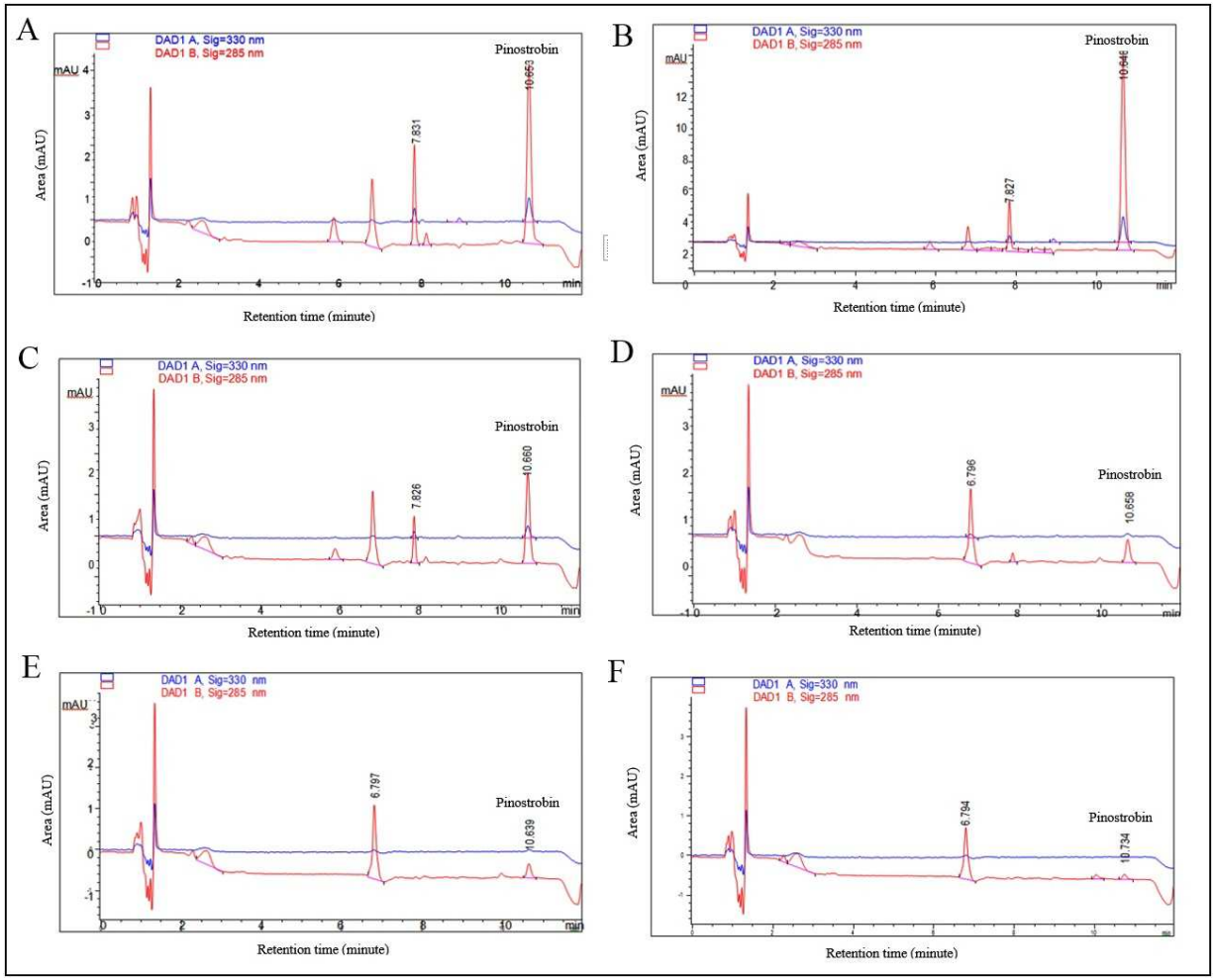

Fig. 2 HPLC chromatogram for pinostrobin content in adventitious roots of *B. rotunda* cultured on (A) 0.25 MS medium, (B) 0.50 MS medium, (C) 0.75 MS medium, (D) 1.00 MS medium, (E) 1.50 MS medium and (F) 2.00 MS medium detected at λ = 330 ( ) and λ = 285 nm ( ) showed pinostrobin was absorbed strongly at λ = 285 nm.

The optimum sucrose concentration for highest production of secondary metabolites may be varied from every plant species [21]. The best sucrose concentration for production of phenolics, flavonoids and chlorogenic acid of *E. angustifolia* root suspension cultures was found to be 5% (w/v) [22] while for *Hypericum perforatum* adventitious root cultures, 5% to 9% (w/v) of sucrose concentration demonstrated the increment of secondary metabolites production [4]. Similar observation also displayed in the present study of adventitious root culture of B. rotunda where the highest pinostrobin content (3.61 ±0.02 µg/g) was enhanced in MS medium supplemented with 5% (w/v) sucrose concentration (Table 2). These results were supported by the detection of pinostrobin content through chromatogram peaks according to the sucrose concentrations treatments using HPLC (Fig. 3).These results may be due to the osmotic stress that occurred during the accumulation of high carbon ion in a nutrient medium that boost the production of secondary metabolite in plant cells [18], [23], [24], [25], [26].

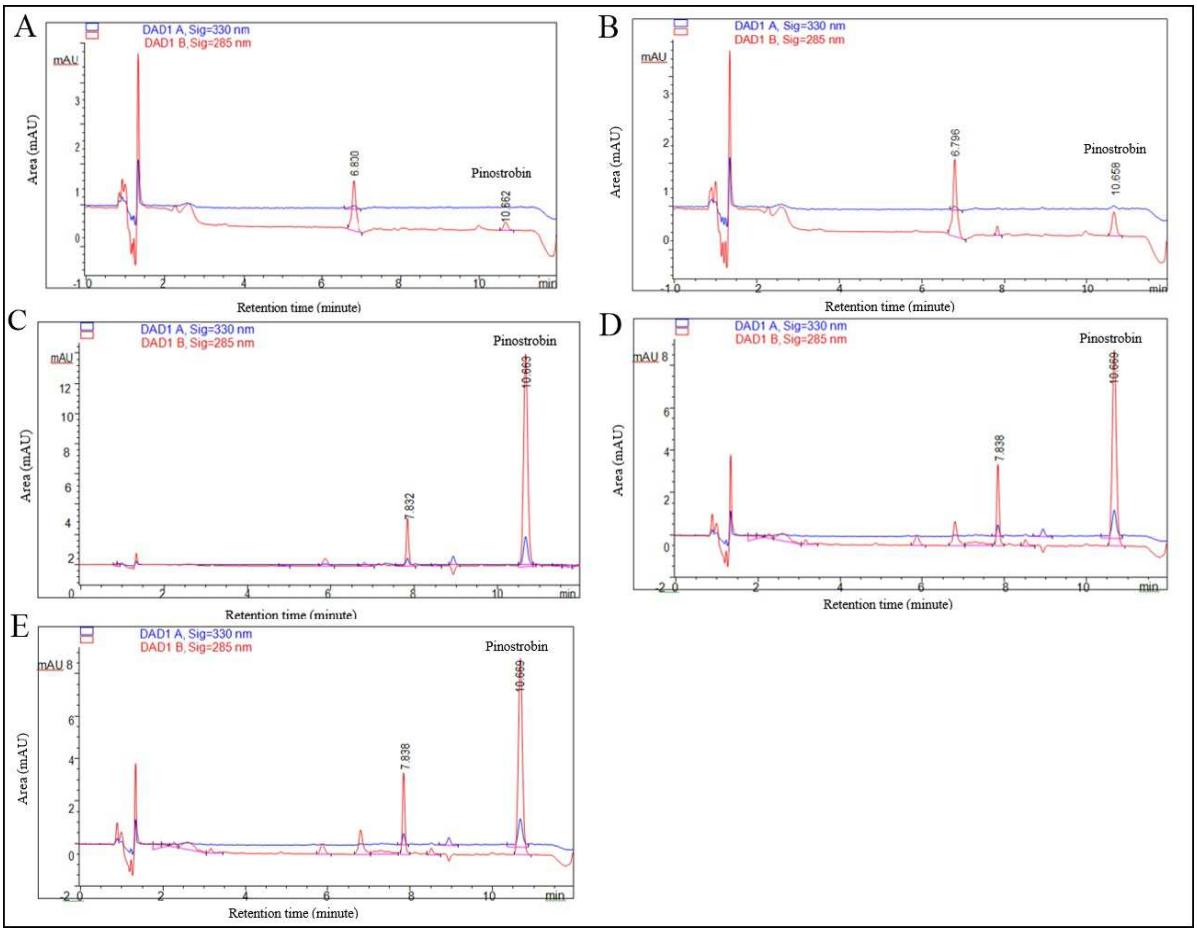

Fig. 3 HPLC chromatogram for pinostrobin content in adventitious roots of *B. rotunda* cultured on MS medium supplemented with (A) 1% sucrose concentration, (B) 3% sucrose concentration, (C) 5% sucrose concentration, (D) 7% sucrose concentration and (E) 9% sucrose concentration detected at λ = 330 ( ) and λ = 285 nm ( ) showed pinostrobin was absorbed strongly at λ = 285 nm.

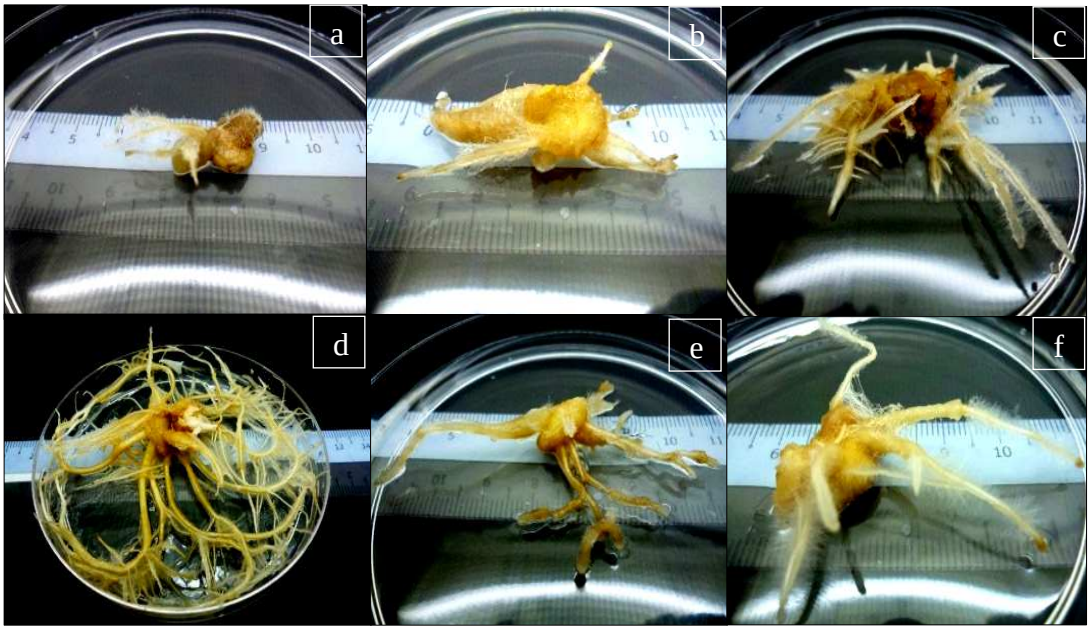

Fig. 4 Adventitious roots produced in different sucrose concentration; (a) 0%; (b) 1%; (c) 3%; (d) 5%; (e) 7%; (f) 9%

IV. Conclusion

HPLC analysis displayed the presence of pinostrobin from the *in vitro* adventitious roots of *B. rotunda*. The growth characteristics (biomass, number of roots and root length) together with pinostrobin content from the established *in vitro* adventitious roots of *B. rotunda* was successfully enhanced by the manipulation of culture conditions involving ½ strength of MS medium and the addition of 5% (w/v) of sucrose. Hence, it is important to study other strategies in order to enhance the maximum production of secondary metabolites in *in vitro* systems.

Nomenclature

centimetre	cm
milimetre	mm
nanometre	nm
microliter	µl
weight per volume	w/v
microgram per gram	µg/g
percentage	%

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