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Effect of basic medium and plant growth regulators on *in vitro* multiplication of *Phaius tankuvillae* (Banks ex L'Heritier de Brutelle) Blume

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The first experiment: effects of basic medium on protocorm like body (PLB) induction was investigated. Buds were cultured on 5 basic media which were Vacin and Went (1949), Thomale GD (1954), Murashige and Skoog (1962), Knudson C (1946) and White (1963). Each medium had added 0.1 mg/l NAA and 0.5 mg/l kinetin. The second experiment: The best liquid basic medium containing a combination of 0, 0.002, 0.004, 0.006, 0.008 and 0.010 mg/l Triacontanol and 0, 0.1, 0.5 and 1.0 mg/l BA was studied. The third experiment: the medium was supplemented with the same concentration of triacontanol and BA as the second experiment except the medium of this experiment was solid. After 16 weeks of incubation, the maximum number of PLB (3.25), the highest percentage of explants produced PLB (75) and the biggest width of explants (1.29 cm.) were obtained from Vacin and Went medium. In the second experiment, after 12 weeks of incubation, the maximum number of PLB (6.75), the highest percentage of explants produced PLB (91.67), the biggest width of explants (1.12 cm.) and the heaviest fresh weight of explants (0.45 g) was achieved from Vacin and Went liquid medium containing 0.004 mg/l Triacontanol and 0.1 mg/l BA. In the third experiment, after 8 weeks of incubation, the maximum number of shoots (7.38), the highest number of PLB (1.50), the biggest width of explants (1.90 cm.), the heaviest fresh weight of explants (1.8 g) and the maximum number of roots (4.37) were also obtained from Vacin and Went solid medium supplemented with 0.004 mg/l triacontanol and 0.1 mg/l BA.

Key words: protocorm like body, Triacontranol, BA, NAA, PLB

Introduction

Phaius tankervillae is a very widespread terrestrial, being found along the eastern seaboard of Australia from well into NSW right up the East coast of Queensland and on through Pagua New Guinea, Indonesia, Malaysia, Vietnam, Thailand and on into Northern India. It has become naturalized on some of the

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islands of the West Indies and ranked as an endangered species in Japan (Hirono *et al.* 2009). The plant is a large one, with stout fleshy psuedobulbs and several large pleated leaves. Infloresence is a simple raceme which may attain 100 cm. hight and bear up to 30 large shapely blooms. The plant is generally propagated through the division of psuedobulbs but the rate of multiplication is very slow. Therefore, an *in vitro* propagation technique could be a useful approach for the mass scale production of this orchid for commercial purposes.

Triacontanol (TRIA), a long 30 carbon primary alcohol, is a naturally occurring plant growth regulator (Ries *et al*., 1977; Ries and Houtz, 1983; Ries and Wert, 1988). TRIA is a component of the epicuticular wax of alfalfa and many other plants (Chibnell *et al*., 1933; Azam *et al*., 1977). Its effect on tissue cultures was tested on *Oryzasativa* (Yun and Kim, 1986), woody plants (Tantos *et al*., 2001).

The aim of this work was to use 5 basic medium, investigation ranges of combinations of triacontanol and BA and effect of solid and liquid medium for protocorm like body and shoot induction.

Materials and methods

Explant source and surface sterilization

Lateral buds were excised from a short bulb-like runner (Figure 1) and were initially shaken in 70% ethanol for 1 min. and then transferred to 0.1 % (W/V) mercuric chloride (plus 2 drops of tween 20) for 15 min. Finally explants were rinsed 3 times in sterile distilled water.

Fig. 1. Lateral buds a short bulb-like runner

Protocorm like bodies (PLBs) initiation

Explant were initially cultured on 5 basic media which were Vacin and Went (1949) (VW), Thomale GD (1954), Murashige and Skoog (1962) (MS), Knudson C (1946) and White (1963). Each medium had added 0.1 mg/l NAA and 0.5 mg/l kinetin. In order to produce the mass of PLB, callus and PLBs were transferred to VW liquid medium (these showed the best results from the first experiment) containing a combination of 0, 0.002, 0.004, 0.006, 0.008 and 0.01 mg/l triacontanol and 0, 0.1, 0.5 and 1.0 mg/l BA. Data were subjected to randomized complete block design, 4 replications and 3 explants per replication.

Shoot induction

The fleshly initiated PLBs were transferred to the solid VW medium supplemented with a combination of 0, 0.002, 0.004, 0.006, 0.008 and 0.01 mg/l triacontanol and 0, 0.1, 0.5 and 1.0 mg/l BA. Data were subjected to 6x4 factorial in randomized complete block design, 24 treatments 4 replication and 3 explants per replication.

Culture conditions

The inoculated culture bottles and flasks were kept in a culture room maintained at a temperature of 25 ± 2ºC photoperiod: 16 h. light and 8 h. dark. Light intensity was maintained at 40 $\mu Em^{-2}s^{-1}$. Liquid cultures were constantly agitated on a rotary shaker at 100 rpm.

Statistic analysis

All data were analysis using ANOVA and Duncan's multiple range tests by SAS programme.

Results and discussions

Explants started to form callus and PLBs within 8 weeks of incubation in Vacin and Went, Murashige and Skoog, and White media VW gave the best percentage of explants formed callus and PLBs (Table 1) while there was no PLB on Thomale GD medium. After 16 weeks of culture, the maximum percentage PLBs (75%), the maximum number of PLB (3.25) and the largest explant size were produced on VW medium. The PLBs were yellow and friable callus were obtained from explant (Figure 2).

Table 1. Effect of 5 basic medium containing with 0.1 mg/l NAA and 0.5 mg/l kinetin on PLB growth 16 weeks of culture

Basic medium	Explant formed PLBs (%)± SE[a]	No. of PLBs± SE[a]	Explant & PLB width ± SE[a]
Vacin and Went	75.00 ± 15.96 a	3.25 ± 0.81 a	1.29 ± 0.14 a
ThomaleGD	0.00 ± 0.00 c	0.00 ± 0.00 c	0.73 ± 0.08 b
Murashige and Skoog	50.00 ± 16.67 ab	2.08 ± 0.48 a	0.85 ± 0.07 b
KnudsonC	33.34 ± 19.25 bc	0.67 ± 0.38 b	0.79 ± 0.08 b
White	16.67 ± 9.62 bc	0.33 ± 0.33 b	0.79 ± 0.10 b
F-test	*	**	**
CV (%)	59.35	16.18	23.37

[a] = Values within a column followed by the same letter are not significance difference by Dancan's multiple range test P<0.05 ,*Significant at P<0.05, **Significant at P<0.01

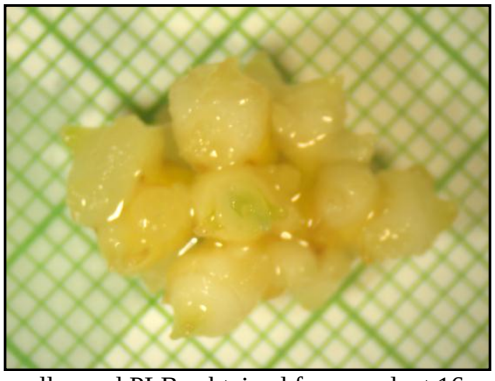

Fig. 2. Friable callus and PLBs obtained from explant 16 weeks of culture

Protocorm like bodies (PLBs) initiation

After 16 weeks of incubation, callus and PLB were transfer to VW medium with a various combination of triacontanol and BA. The medium with 0.004 mg/l triacontanol and 0.1 mg/l BA gave the best percentage of explant formed PLBs. In addition, the number of PLBs and explant weight also achieved from this medium (Table 2). Explant started to form PLBs within 4 weeks of incubation. The explant remained green and change to yellow within 6-8 weeks. (Figure 3) and increased the number of PLBs within 10 weeks (Figure 4). According to Ravindra *et al*., 2005, triacontanol 0.004 mg/l gave the highest percentage of explant formed PLBs in *Dendrobium nobile*. Similarly, Biernbum *et al*. (1998) said that the low concentration of triacontanol was better than high concentration.

Table 2. Effect of Tricontanol and BA in VW liquid medium on PLBs growth 12 weeks of culture

Triacontanol (mg/l)	BA (mg/l)	Explant formed PLBs (%) ± SE[a]	No. of PLBs ± SE[a]	Explant & PLBs weight (g) ± SE[a]
0	0	0.00 ± 0.00	0.00 ± 0.00	0.08 ± 0.03
	0.1	0.00 ± 0.00	0.00 ± 0.00	0.11 ± 0.03
	0.5	8.33 ± 8.33	0.25 ± 0.25	0.23 ± 0.09
	1.0	0.00 ± 0.00	0.00 ± 0.00	0.14 ± 0.05
0.002	0	25.00±8.33	0.50 ± 0.29	0.18 ± 0.05
	0.1	0.00 ± 0.00	0.00 ± 0.00	0.25 ± 0.11
	0.5	16.67 ± 9.62	0.17 ± 0.17	0.15 ± 0.51
	1.0	8.33 ± 8.33	0.25 ± 0.25	0.09 ± 0.07
0.004	0	50.00 ± 21.25	1.83 ± 1.52	0.29 ± 0.12
	0.1	91.67 ± 8.33	6.75 ± 4.21	0.45 ± 0.14
	0.5	33.33 ± 13.61	0.92 ± 0.63	0.13 ± 0.03
	1.0	16.67 ± 9.62	0.84 ± 0.48	0.08 ± 0.02
0.006	0	8.33 ± 8.33	0.50 ± 0.32	0.18 ± 0.07
	0.1	16.67 ± 9.62	0.42 ± 0.25	0.05 ± 0.12
	0.5	8.33 ± 8.33	2.58 ± 2.58	0.19 ± 0.06
	1.0	8.33 ± 8.33	0.33 ± 0.33	0.22 ± 0.09
0.008	0	16.67 ± 16.67	0.50 ± 0.50	0.07 ± 0.02
	0.1	8.33 ± 8.33	0.34 ± 0.19	0.13 ± 0.06
	0.5	8.33 ± 8.33	0.25 ± 0.05	0.20 ± 0.17
	1.0	8.33 ± 8.33	0.00 ± 0.00	0.17 ± 0.07
0.01	0	8.33 ± 8.33	0.33 ± 0.33	0.05 ± 0.01
	0.1	16.67 ± 9.62	1.00 ± 0.64	0.12 ± 0.04
	0.5	16.67 ± 9.62	0.34 ± 0.19	0.05 ± 0.01
	1.0	8.33 ± 8.33	0.17 ± 0.17	0.07 ± 0.02
F-test		ns	ns	ns
CV (%)		116.75	35.06	5.92

[a] = Values within a column followed by the same letter are not significance difference by Dancan's multiple range test P<0.05, ns = non-significant, [*]Significant at P<0.05

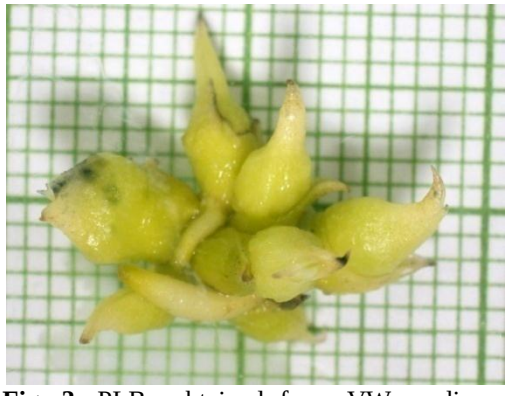

Fig. 3. PLBs obtained from VW medium containing 0.004 mg/l triacontanol and 0.1 mg/l BA 6-8weeks of culture

Fig. 4. PLBs obtained from liquid VW medium containing 0.004 mg/l triacontanol and 0.1 mg/l BA 10weeks of culture

Shoot induction

To induce shoots, callus and PLBs derived were transferred to solid VW medium with the same combinations of triacontanol and BA to the liquid medium. The medium with 0.004 mg/l triacontanol and 0.1 mg/l BA still showed the best production of shoots (PLB formed shoot and the number of shoots) (Table 3) (Figure 5) and the maximum number of roots also obtained from this medium (Table 3). This was supported by Fratenale *et al*. (2003), the maximum number of shoots of *Thymus mastichina*were achieved on medium with 0.1 mg/l BA and 0.002–2 mg/l triacontanol.

Table 3. Effect of Tricontanol and BA in VW solid medium on shoot growth 8 weeks of culture

Triacontanol (mg/l)	**BA (mg/l)**	**Explant & PLBs formed shoot± SE[a]**	**No. of shoots ± SE[a]**	**No. of roots ± SE[a]**
0	0	66.67 ± 13.61	1.00 ± 0.00	0.50 ± 0.50 b
	0.1	33.34 ± 0.00	1.00 ± 0.00	0.00 ± 0.00 b
	0.5	25.00 ± 15.96	1.50 ± 0.65	1.75 ± 1.75 ab
	1.0	3.34 ± 19.25	1.00 ± 0.58	2.50 ± 1.89 ab
0.002	0	25.00 ± 8.33	2.25 ± 1.60	0.00 ± 0.00 b
	0.1	25.00 ± 15.96	0.50 ± 0.29	0.00 ± 0.00 b
	0.5	25.00 ± 15.96	0.75 ± 0.48	0.50 ± 0.50 b
	1.0	16.67 ± 9.62	0.25 ± 0.25	0.00 ± 0.00 b
0.004	0	33.34 ± 0.00	4.50 ± 2.53	3.25 ± 0.70 ab
	0.1	75.00 ± 8.33	7.63 ± 2.79	4.38 ± 0.25 a
	0.5	58.33 ± 15.96	1.67 ± 0.41	1.67 ± 1.67 ab
	1.0	41.67 ± 15.96	0.88 ± 0.31	0.50 ± 0.50 b
0.006	0	41.67 ± 15.96	1.25 ± 0.48	3.25 ± 1.97 ab
	0.1	25.00 ± 8.33	0.75 ± 0.25	0.00 ± 0.00 b
	0.5	25.00 ± 8.33	1.25 ± 0.25	0.00 ± 0.00 b
	1.0	33.34 ± 13.61	0.75 ± 0.25	0.50 ± 0.50 b
0.008	0	41.67 ± 8.34	1.13 ± 0.13	1.00 ±1.00 b
	0.1	16.67 ± 9.62	1.25 ± 0.95	1.50 ± 1.50 ab
	0.5	41.67 ± 8.34	0.63 ± 0.38	0.25 ± 0.25 b
	1.0	33.34 ± 13.61	0.75 ± 0.25	0.38 ± 0.38 b
0.01	0	16.67 ± 9.62	0.50 ± 0.29	0.50 ± 0.50 b
	0.1	25.00 ± 8.33	2.75 ± 1.55	2.25 ± 1.44 ab
	0.5	16.67 ± 9.62	0.50 ± 0.29	0.00 ± 0.00 b
	1.0	25.00 ± 15.96	0.63 ± 0.38	0.00 ± 0.00 b
F-test		ns	ns	*
CV (%)		66.76	28.92	37.98

[a] = Values within a column followed by the same letter are not significance difference by Dancan's multiple range test P<0.05, ns = non-significant, * Significant at P<0.05

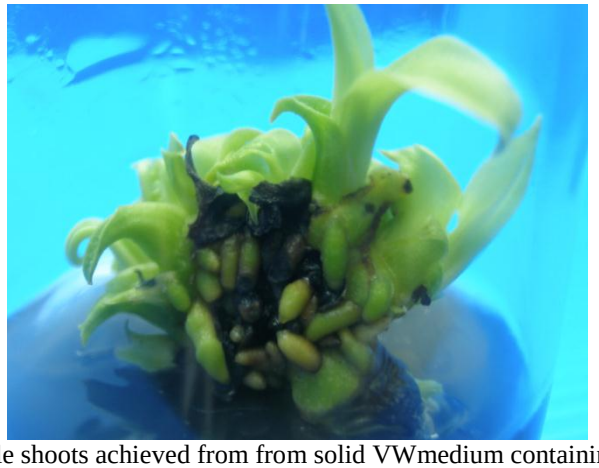

Fig. 5. Multiple shoots achieved from from solid VWmedium containing 0.004 mg/l triacontanol and 0.1 mg/l BA 8weeks of culture

Conclusion

In this study was investigated basic medium for PLB production. The highest percentage of explants produced PLB were obtained from VW medium. The liquid VW medium containing 0.004 mg/l Triacontanol and 0.1 mg/l BA gave the maximum number of PLB, the highest percentage of explants produced PLB, and the heaviest fresh weight of explants. The maximum number of shoots and roots were also obtained from VW solid medium supplemented with 0.004 mg/l triacontanol and 0.1 mg/l BA.

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