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A Protocol Towards Micropropagation of the Piscicidal Plant, *Maesa ramentacea* A. DC.

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ABSTRACT A tissue culture system was established from shoot tips of the piscicidal plant, *Maesa ramentacea* A. DC. Shoot proliferation was achieved with Murashige and Skoog (MS) medium supplemented with either BA, BA-Kinetin or BA-NAA combinations. Stocking of shoot cultures was obtained by repeating subculture in the same medium at 30-day intervals wherein the rate of multiplication was maintained. For rooting, the individual shoot was implanted on root induction medium consisted of MS medium supplemented with 2 mg/l NAA. Within two weeks of incubation 100% rooting was evidenced. Complete plantlets were acclimatized and successfully transplanted to soil.

KEYWORDS: *Maesa ramentacea* A. DC., micropropagation, multiple shoots, piscicidal plant, tissue culture.

INTRODUCTION

Maesa ramentacea A. DC. (Family Myrsinaceae) is an evergreen shrub or small tree. Resin cavities and resin ducts are found in the leaves, pith, wood and bark[1]. Tuntiwachwuttikul *et al.*[2] reported that the leaves of many *Maesa* spp. are a rich source of quinones, flavonoids, triterpenoids and saponins. An aqueous extract of the leaves of *Maesa ramentacea* A. DC. was found to show good piscicidal activity (LD_{100} = 100 mg/l). They isolated and elucidated a new triterpenoid saponin from the leaves of *Maesa ramentacea* A. DC. These leaves could be potentially used as a substitute for tea seed cake as a fish poison in commercial fish and prawn farming. The advantage of using these compounds as a piscicidal is that there is no side effect to human and environment.

In nature, propagation of this plant is by seed, hence wide variation in the field is common due to heterozygosity of the seedlings and conventional vegetative propagation is encompassed with problems of poor seed viability and low germination. There is an urgent need to apply non- conventional propagation methods for conservation and large scale delivery of *Maesa ramentacea* A. DC.

In the present paper, we report on rapid clonal propagation through axillary bud break in the shoot tip culture and field establishment of uniform plant of *Maesa ramentacea* A. DC.

Authors pls provides running title:-

MATERIALS AND METHODS

Plant material - 3-month old selected seedlings were used as source of explants. After initial washing thoroughly in running tap water for 3-5 minutes, shoot tips (1.5-2 cm) were excised and surface sterilized by either immersing in 20% (v/v) Clorox aqueous solution for 20-30 min or 0.1g/l mercuric chloride for 10-15 min. The explants were then rinsed several times with sterile distilled water to remove the trace of sterilant. Shoot tips with 2-3 leaf pairs were implanted vertically into the medium.

Nutrient media - Murashige and Skoog[3] (MS) medium containing 30 g/l sucrose and gelled with1.5 g/l Gelrite (Merck & Co., Kelco Division, NJ, USA) served as the basal medium. The media were adjusted to a pH of 5.7 with 0.1 N NaOH or HCl before adding agar and autoclaved at 121°C and 1.05 kg/cm^2 for 20 min.

For shoot multiplication, 6-benzyladenine (BA) at different concentrations (1, 3, 5, 7 mg/l) was used. Kinetin (0.5, 1, 1.5, 2 mg/l) was used in combination with BA held constant at 3 mg/l. For root initiation, single shoot with 3-5 cm length and 4-5 leaves excised from multiple shoots was inoculated in the basal medium supplemented with 2 mg/l each of 1-naphthaleneacetic acid (NAA) and 3-indolebutyric acid (IBA). Since auxins and cytokinins were effective in plantlet formation, these experiments were conducted to determine in different combinations using NAA (1, 2, 3 mg/l) and BA (1,

3, 5, mg/l).

Culture conditions - Explants were inoculated in 115 ml screw-topped jars each containing 20 ml of medium. In all experiments, the cultures were incubated at 25±2°C with a 16-h photoperiod under an illumination of 20 $\mu molm^{-2}s^{-1}$ photosynthetic photon flux density provided by Sylvania Gro-lux lamps. Five explants were planted per culture, at least 10 cultures were raised for each treatment and all experiments were repeated three times.

Results

It was found that shoot tips (1.5-2 cm) with 2-3 leaf pairs which were surface sterilized in 20% Clorox aqueous solution for 20 and 30 min gave 30 and 45% uncontaminated tissues, respectively. While 0.1g/l mercuric chloride for 10 and 15 min provided 70 and 80% uncontaminated tissues; however, damaged tissues were evident with 15 min treatment with 0.1g/l mercuric chloride (Table 1). Therefore we routinely used 0.1g/l mercuric chloride for 10 min for the sterilization of explants.

After initial culture for 14 days in MS medium lacking growth regulators, no morphogenetic responses were observed. Leaves were green with only slight enlargement. Activation of the shoot tips took place with the plant growth regulators added to the basal medium. BA was individually employed since BA itself was effective on multiple shoot formation. The results revealed that at all concentrations of BA tested, multiple shoots developed without the intervening callus stage within 20 days of culture (Fig 1a). The number of shoots was dependent on concentrations of BA. The best being 3 mg/l BA with the average number of 48 shoots per explant was obtained (Fig 1b). After initial culture for 50 days, these small numerous shoots expanded thereafter they were dissected and subcultured onto the same medium. Subsequently, mass propagation of shoots was achieved by repeating subculture at 30-day intervals. Prolonged culture in the same medium resulted in better shoot growth, therefore attempts were made to induce maximum growth by recultures in a medium containing reduced levels of BA i.e. from 7 mg/l BA to 1 mg/l BA. These results showed that the lower BA concentration had a better growth of stems and leaves. Moreover, if BA was omitted from the medium, many thin and long roots were observed (Fig 2).

To study the synergistic effect, Kinetin was selected as the representative for this purpose. Among the combinations used, 3 mg/l BA and 2 mg/l Kinetin was favorable since the highest shoot number was obtained in 40 days after culture (Fig. 3a). It was found that BA alone was more effective in inducing shoots than all combinations of BA and Kinetin. However, shoots and leaves grew vigorously in BA and Kinetin containing medium (Fig. 3b). In addition, some of the plantlets thus obtained developed tiny and long roots.

Although the previous finding revealed that rooting could be obtained in the medium without exogenous auxins, the roots were brittle and the plants with such roots showed poor establishment after hardening. Hence shoot cuttings (3-5 cm height) were excised from proliferating cultures and used for rooting studies. IBA and NAA (2 mg/l each) were incorporated in the basal medium. Root induction was stimulated in both media (Table 2). More roots developed from the excised shoots on

Table 1. Sterilization of *Maesa* shoot tips using commercial Clorox and mercuric chloride at various times.

Sterilants	Time (min.)	% Uncontanimation	Visual observation
20% Clorox	20	30	green
	35	45	brown
0.1g/l $HgCl_2$	10	70	green, healthy
	15	80	brown, necrotic

Table 2. The influence of IBA and NAA on the root initiation of *Maesa*.

Auxins (2 mg/l)	Root number /explant (X±SD)	Root length (cm) /explant (X±SD)	Visual observation
IBA	22.0±0.1	0.4±1.3	short, thin
NAA	18.0±0.1	2.3±1.1	thick, long and fibrous

Table 3. Effect of NAA at various concentrations on the root initiation after culture *in vitro* for 2 weeks.

NAA (mg/l)	Root number /explant (X±SD)	Root length (cm) /explant (X±SD)	Visual observation
1	12.0±0.1	2.8±1.1	short, thin
2	17.0+0.1	3.4±1.0	thick, long and fibrous
3	7.0+0.1	3.8±0.8	short, thin
4	8.0±0.1	2.7±1.4	short, thin

basal media containing IBA than those containing NAA; however, roots that developed from shoots in the presence of NAA were thick, long and fibrous (Fig 4). Consequently, an experiment was initiated to assess the effectiveness of NAA at different concentrations (Table 3). At 2 mg/l NAA, rooting occurred with a high root number and relatively sufficient root length. Two weeks of rooting incubation were adequate before transplanting into soil.

In achieving both shoot and root regeneration simultaneously, an experiment was conducted over a series of BA-NAA ratios. Culturing the shoots on media supplemented with BA and NAA evoked morphogenetic responses. The data are presented in Table 4. Multiple shoots and roots were observed at all combinations. Rooting was not preceded by the formation of callus. The highest shoot number and shoot length were obtained with 1 mg/l BA and 2 mg/l NAA. Number of roots in this medium was lower than those in the medium with 5 mg/l BA and 1 mg/l NAA but the root length was longer.

When the *in vitro* complete plantlets reach a length of 6-7 cm, they were transferred to small black plastic bag containing sterile vermiculite. These plantlets were covered with transparent plastic sheet and watered twice daily. Later they were transplanted in a potting mixture of sand, manure and decayed leaf (1:1:1) and hardened in a mist bed with 100% survival (Fig 5). It is calculated that starting from one shoot tip provided ca. 45-50 shoots (Fig 1), each of which could produce up to 150 shoots in 3 months for a total production of 1650-7500 plantlets per explant in five months. Regenerants did not display any phenotypic variation during subsequent vegetative and floral development. Cytological studies of the tissue culture-derived plants are under investigation.

Discussion

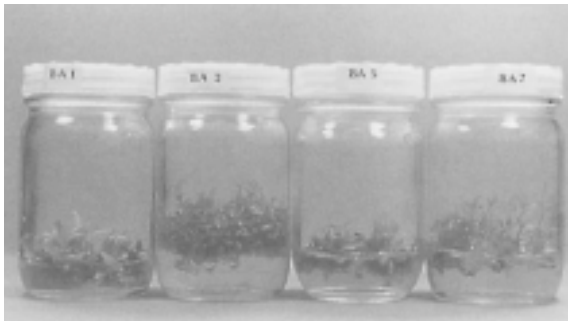
Contamination has been found to be a considerable problem in *Maesa* tissue culture. By using 0.1g/l mercuric chloride for 10 min this problem was overcome and found to be satisfactory since the sterilization procedure yielded 70% aseptic cultures.

Our results demonstrated that a system to regenerate shoots of *Maesa* has been developed. Shoot tips appeared to be a potentially suitable explant for micropropagation since as many as 48 shoots per culture were obtained with 3 mg/l BA. An interesting observation made in the present study in cultured explant of *Maesa* concerned the effect of BA, BA-Kinetin, and BA-NAA interaction. In the present study, our result was in concurrence with *Lilium*;[4] *Citrullus*;[5] *Sinningia*;[6] *Zephyranthes*[7] when BA was supplied as a sole growth regulator to promote shoot organogenesis. Shoot multiplication was not observed on growth regulator-free medium. This result suggested that the normal endogenous growth substance levels were not conductive to bud formation. An increase in multiple shoots was clearly seen with exogenous supply of BA in the media suggested that bud formation required cytokinin. This also indicated the existence of totipotent cells in this region which can be activated with an appropriate concentration of cytokinin.[8]

A conjunction of BA and Kinetin did not evoke a better response in shoot multiplication than BA alone. This is probably due to the difference endogenous levels of growth regulators in this plant or to a difference in sensitivity.[9] In contrast, the remarkable difference in organogenesis and subsequent plantlet formation was found in BA-NAA interactions suggested the synergistic effect. This

Table 4. Effect of NAA and BA concentrations on plantlet formation in *Maesa* after culture *in vitro* for 3 weeks.

MS medium		Shoot number /explant (X±SD)	Shoot length (cm)/explant (X±SD)	Root number /explant (X±SD)	Root length (cm)/explant (X±SD)
BA (mg/l)	NAA (mg/l)				
1	1	6.0±0.2	4.7±0.2	6.0±0.2	2.5±0.2
1	2	18.0±0.1	3.4±0.2	4.0±0.1	3.3±0.2
1	3	2.0±0.2	4.4±0.2	7.0±0.2	1.2±0.2
3	1	10.0±0.2	5.9±0.1	2.0±0.2	0.4±0.1
3	2	12.0±0.2	4.2±0.2	5.0±0.1	1.1±0.2
3	3	18.0±0.2	2.5±0.2	6.0±0.2	1.9±0.1
5	1	10.0±0.2	7.6±0.2	18.0±0.1	2.6±0.1
5	2	6.0±0.2	3.7±0.1	15.0±0.2	1.5±0.2
5	3	3.0±0.2	6.3±0.2	13.0±0.1	0.8±0.2



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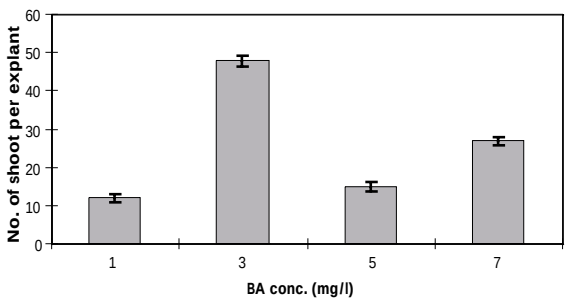

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Fig 1. (a) *Maesa* multiple shoots cultured on MS medium containing 1, 3, 5, 7 mg/l BA for 20 days (x125).
(b) Effect of BA on shoot number production of *Maesa* cultured on the same medium as in (a). Each value represents the mean ± S.D.

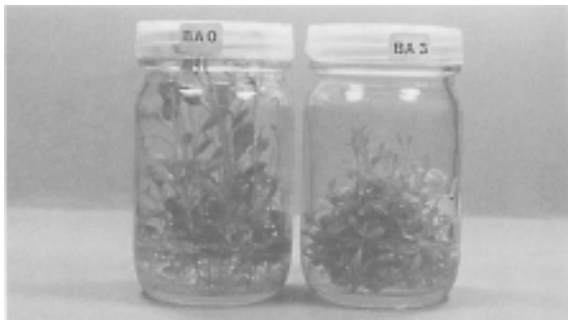
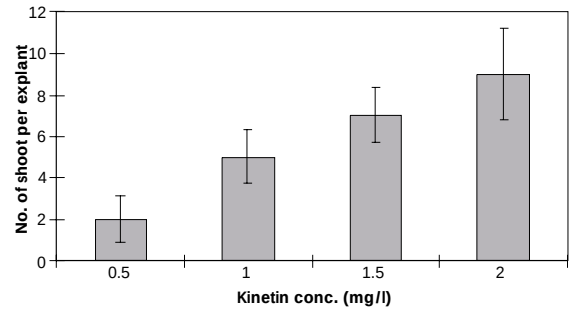


Fig 2. Root growth in *Maesa* occurred when multiple shoots were transferred from MS containing 3 mg/l BA (right) to medium devoid of BA (left) without the addition of auxins (x165).



a

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Fig 3. (a) The effect of MS medium supplemented with 3 mg/l BA in combinations with 0.5, 1, 1.5, and 2 mg/l Kinetin on multiplication of *Maesa* for 40 days. Each value represents the mean ± S.D.
(b) Development of shoots cultured on MS medium containing 0.5, 1, 1.5, and 2 mg/l Kinetin (from left to right). Better shoot growth was found in the medium containing lower concentrations of Kinetin (x125).

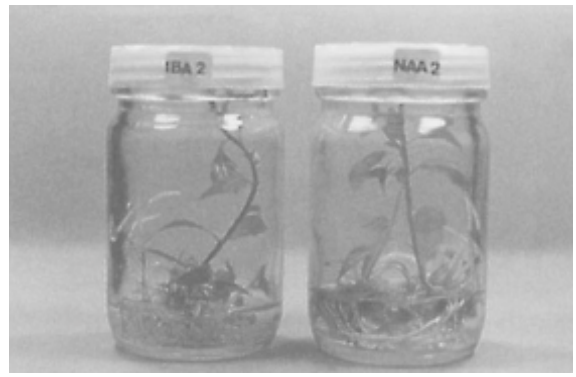

Fig 4. Isolated shoots on MS medium supplemented with 2 mg/l IBA and 2 mg/l NAA showing root formation. Noted that roots occurred in NAA containing medium were thicker and longer (x165).

Fig 5. Complete *Maesa* plantlets established in potting soil (x165).

result revealed that it is possible to obtain plantlets on one medium without the necessity of transferring the shoots to another medium for rooting.

Obviously, regenerated shoots can be rooted readily providing a rapid clonal propagation. Root regeneration was found when BA was reduced by prolonged culture or omitted from the medium. In addition, roots obtained from NAA containing medium showed better characters than those from IBA suggested that the cellular sensitivity to a particular growth regulators plays a significant role in determining the pattern of regeneration.[10]

In conclusion, this study reported the induction of plantlets by culturing shoot tips of *Maesa*. The obtained plantlets are accounted for genetic stability thus reducing somaclonal variation production and hold a great promise for its large scale clonal propagation.

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