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Callus Induction and Plant Regeneration in African Marigold (*Tagetes erecta* L.)

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Pretreatment of hypocotyl and leaf explants of African marigold (*Tagetes erecta* L.) with 1.0% ascorbic acid significantly reduced excessive browning of excised explant tissue. This method together with interval transfer of calli onto fresh culture medium was effective in preventing further tissue browning of calli cultured on MS medium supplemented with various concentrations and combinations of naphthalene acetic acid (NAA) and 6-benzyladenine (BA). Response of the explants from the two organs varied in terms of callus formation and plant regeneration. Plantlet regeneration from hypocotyl-derived calli occurred on MS medium containing 0.2 to 1.0 mg/*l* NAA and 0.5 to 5.0 mg/*l* BA, whereas leaf-derived calli produced mainly roots and green spots in some plant growth regulator combinations. The leaf explants also produced large yellow calli on MS medium containing 5.0 mg/*l* NAA and 0.2 to 5.0 mg/*l* BA.

KEY WORDS: *Tagetes erecta,* marigold, callus, plant regeneration, hypocotyl.

Introduction

African marigold (*Tagetes erecta* L.) reduces the population level of the root-lesion nematode (*Pratylenchus penetrans* COBB.) in the soil (OBAYASHI 1989; UHLENBROEK and BIJLOO 1958). The nematocidal effect is attributed to the presence of thiophenes (CHAN *et al.,* 1975), which are naturally occurring biocides (CROES *et al.,* 1989a and b; GOMMERS *et al.,* 1982; HULST *et al.,* 1989). Thiophenes can be extracted by polar solvents (HATAKEDA *et al.,* 1985, HULST *et al.,* 1989) or through a bioreactor (CROES *et al.,* 1988; KETEL 1986) and can be quantified even in minute amount (CROES *et al.,* 1988). However, the actual application of thiophenes as a biocide has not yet been realized presumably due to its low production from calli and/or through a bioreactor. Thiophene content also decreases in highly root-regenerating calli (KETEL 1986; WIERMANN 1981; YEOMANN *et al.,* 1980). As an alternative approach, African marigold can be utilized as a source of nematode-resistance genes for incorporation into economically important crops. Protoplast fusion which results in the production of somatic hybrids between distantly related species (DUDITS *et al.,* 1979), offers a unique method of incorporating useful genes into cultivated crops (MELCHERS 1977). Somatic hybrids afford new gene combinations that have not been possible through sexual hybridization. However, the successful application of this technique is markedly dependent upon the efficient plant regeneration in cell and tissue culture of marigold.

Previous studies have been mainly focused on the accumulation and extraction of thiophenes from callus and cell cultures (CROES *et al.,* 1989a, 1989b; HULST *et al.,* 1989; KETEL 1986, 1987). Moreover, consecutive studies by KETEL *et al.* (1985) and KETEL (1986 and 1987) revealed severe browning and stoppage of growth of tertiary leaf-derived calli of *Tagetes patula* and, primary and secondary leaf-derived calli of *T. erecta,* respectively. In addition, CROES *et al.* (1989a) reported the formation of only roots from stem-derived calli of *T. minuta.* The

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above-mentioned studies point to the difficulty in callus formation and plant regeneration in some marigold species. So far, there has been no report on plant regeneration from callus of marigold. This paper describes a simple method for preventing excessive browning of tissue explant, and outlines an efficient procedure for callus formation and plant regeneration from hypocotyl-derived calli, and callus formation from leaf explants of African marigold (*Tagetes erecta* L.).

Materials and Method

Seeds of African marigold "African tall" (*Tagetes erecta* L.) from Sakata Seed Co. Ltd. were surface-sterilized with 1.0% sodium hypochlorite solution added with two to three drops of Tween 20 for 15 min., and then washed three times with sterile distilled water. This procedure was repeated twice. The sterilized seeds were sown in growth regulator-free MS medium (MURASHIGE and SKOOG 1962) containing 30.% sucrose and solidified with 0.8% agar at pH 5.8. Within three weeks, hypocotyl (1 cm) and leaf (1 cm^3) explants were taken from *in vitro*-germinated plants. Prior to inoculation, the hypocotyl and leaf explants were dipped in 1.0% filter-sterelized ascorbic acid through 0.20 millipore microfilter for one minute to reduce browning of the excised tissue. MS medium supplemented with 3.0% sucrose, 0.8% agar and 0.2, 0.5, 1.0, 5.0 mg/*l* naphthalene acetic acid (NAA) and 0.2, 0.5, 1.0, 5.0 mg/*l* 6-benzyladenine (BA) in all combinations were used for callus induction. The pH of the culture media was adjusted to 5.8 prior to autoclaving. Cultures were incubated in continous diffused light of 1,000 lux at 27 ± 1°C. Hypocotyl-derived calli were transferred to fresh medium at one week interval to reduce the browning of explants. These transfers were more efficient in preventing browning than the addition of activated charcoal into the culture medium as shown in the preliminary experiment. Contrastingly, slight or no browning occurred in leaf-derived calli so that there was no need for frequent transfer to fresh culture medium. After three weeks, leaf- and hypocotyl-derived calli were transferred onto growth regulator-free MS medium for plant regeneration.

Results and Discussion

Certain marigold species i.e. *T. erecta* and *T. patula* are difficult to culture *in vitro* due to browning of explant that led to poor callus growth or death of explants KETEL *et al.* (1985) and KETEL (1986 and 1987). Blackening of explants was reported to be due to the oxidation of phenol-like substances (WIERMANN 1981) and other growth inhibiting substances that are excreted into the culture medium, causing the gradual browning of the agar medium (JOHANSSON *et al.* 1982). Tissue browning can be very severe in some crops such as potato, yam, abaca, etc. (AMMIRATO 1982). To eliminate or minimize this phenomenon, various methods were adopted which included soaking the explants in sterile distilled water (AMMIRATO 1982) and incorporating antioxidants e.g. cysteine (NG and HAN 1985) in the culture medium. However, pretreatment of hypocotyl and leaf explants of marigold with 1.0% ascorbic acid prior to inoculation onto the culture medium together with interval transfer of explants or calli onto fresh culture medium significantly reduced or eliminated the browning of tissue and agar medium. This method is very simple, easy and efficient for the

prevention of browning and can sustain or enhance good callus formation and growth.

The formation of calli, green spots, roots and plantlet from hypocotyl and leaf explants of African marigold "African tall" is shown in Tables 1 and 2. Both hypocotyl and leaf explants developed calli at various degree on MS media containing 0.2 mg/*l* NAA and 5.0 mg/*l* BA (media Nos. 1-20 and 2-20). The fine-textured calli were pale yellow in hypocotyl and dark yellow in leaf explants. Leaf-derived calli turned white to brown after three weeks and hence were transferred onto fresh culture medium.

Within two weeks after culture, both hypocotyl- and leaf-derived calli developed roots at

Table 1. Callus formation and plant regeneration from hypocotyl explants of *Tagetes erecta*, marigold "African tall"[1].

Medium No.	NAA (mg/*l*)	BA (mg/*l*)	Callus size[2]	Callus color[3]	% Calli[4] with green spots	% Calli[4] with plantlet	% Calli[4] with roots	Number of roots[5]
1- 1	0.2	0.2	+	Br	0.0	0.0	100.0	+ +
1- 2	0.2	0.5	+	Br	0.0	0.0	93.3	+
1- 3	0.2	1.0	+	PY, Br	0.0	0.0	83.3	+
1- 4	0.2	2.0	+	PY, Br	36.7	30.0	50.0	+
1- 5	0.2	5.0	+ +	PY, Br	33.3	16.7	30.0	+
1- 6	0.5	0.2	+ +	PY, Br	16.9	0.0	100.0	+ + +
1- 7	0.5	0.5	+	W	0.0	0.0	96.7	+ +
1- 8	0.5	1.0	+ +	PY, W	0.0	30.0	53.3	+
1- 9	0.5	2.0	+ +	Br	0.0	30.0	60.0	+
1-10	0.5	5.0	+ +	PY, Br	36.7	36.7	30.0	+
1-11	1.0	0.2	+	PY	0.0	0.0	96.7	+
1-12	1.0	0.5	+ + +	PY	0.0	50.0	100.0	+ + +
1-13	1.0	1.0	+ + +	Br	0.0	30.0	90.0	+ + +
1-14	1.0	2.0	+	Br	0.0	0.0	56.7	+
1-15	1.0	5.0	+ +	PY, Br	80.0	0.0	13.3	+
1-16	2.0	0.2	+	W	0.0	0.0	100.0	+ + +
1-17	2.0	0.5	+	W	0.0	0.0	83.3	+ +
1-18	2.0	1.0	+	W	0.0	0.0	93.3	+ +
1-19	2.0	2.0	+	W	0.0	0.0	80.0	+
1-20	2.0	5.0	—	—	0.0	0.0	0.0	—
1-21	5.0	0.2	+ +	PY	0.0	0.0	100.0	+ +
1-22	5.0	0.5	+ +	PY	0.0	0.0	100.0	+
1-23	5.0	1.0	+	W	0.0	0.0	80.0	+
1-24	5.0	2.0	+ + +	PY, W	0.0	0.0	63.3	+
1-25	5.0	5.0	+	B, W	13.3	0.0	0.0	—

1) Murashige and Skoog's (MS) basal medium with 30.0g/*l* sucrose and 8.0g/*l* agar at pH 5.8.
2) —: no callus, +: ⩾3 mm, + +: 4～9 mm, + + +: >10 mm in diameter
3) Y: yellow, PY: pale yellow, W: white, Br: brown, B: black
4) Thirty explants from each hypocotyl and leaf were cultured for each treatment combination of NAA and BA.
5) —: no root, +: ⩾10, + +: 11～29, + + +: >30 roots/callus

various degree as reported by KETEL (1986 and 1987) except on the media Nos. 1-20, 1-25 and 2-20. Generally, high concentrations of BA (5.0 mg/*l*) reduced the incidence of rooting, while low concentrations of BA (0.2 to 0.5 mg/*l*) induced the formation of calli which developed roots as observed by BOLLMARK *et al.* (1988) and SKOOG and MILLER (1957).

Plant regeneration occurred only from hypocotyl calli (Fig. 1a) cultured on media nos. 1-4, 1-5, 1-8, 1-9, 1-10, 1-12 and 1-13, in the range of 16.7% (No. 1-5) to 50% (No. 1-12). Some of these plantlets developed from green spots (Nos. 1-5, 1-10, 1-15) after transfer of the materials to growth regulator-free MS medium. Meanwhile, leaf-derived calli produced mostly roots, which confirmed the report by KETEL (1987) with occasional green spots in the media nos. 2-5, 2-9 and 2-10.

The results of this study showed that pretreatment of explants with 1.0% ascorbic acid is effective in reducing excessive browning of tissue. This method together with interval

Table 2. Callus and root formation from leaf explant of *Tagetes erecta,* marigold "African tall".

Medium	NAA (mg/*l*)	BA (mg/*l*)	Callus		% Calli with			Number of roots
			size	color	green spots	plantlet	roots	
2- 1	0.2	0.2	+	PY, Br	0.0	0.0	90.0	+
2- 2	0.2	0.5	+	PY, Br	0.0	0.0	83.3	+
2- 3	0.2	1.0	+	Br	0.0	0.0	73.3	+
2- 4	0.2	2.0	+	PY, Br	0.0	0.0	66.7	+
2- 5	0.2	5.0	+	PY, Br	13.3	0.0	0.0	+
2- 6	0.5	0.2	+	PY	0.0	0.0	90.0	+ +
2- 7	0.5	0.5	+	PY	0.0	0.0	96.7	+ +
2- 8	0.5	1.0	+	PY, Br	13.3	0.0	83.3	+
2- 9	0.5	2.0	+ +	PY, Br	10.0	0.0	66.7	+
2-10	0.5	5.0	+ +	PY, Br	13.3	0.0	53.3	+
2-11	1.0	0.2	+	W	0.0	0.0	100.0	+
2-12	1.0	0.5	+ +	Y, Br	0.0	0.0	100.0	+
2-13	1.0	1.0	+ +	Y, Br	0.0	0.0	100.0	+
2-14	1.0	2.0	+ +	Y, Br	0.0	0.0	100.0	+
2-15	1.0	5.0	+ +	Y, Br	0.0	0.0	100.0	+
2-16	2.0	0.2	+ +	PY	0.0	0.0	96.7	+ + +
2-17	2.0	0.5	+ +	PY	0.0	0.0	100.0	+ + +
2-18	2.0	1.0	+	Br	0.0	0.0	100.0	+ + +
2-19	2.0	2.0	+ +	PY, Br	0.0	0.0	93.3	+ + +
2-20	2.0	5.0	—	—	0.0	0.0	0.0	—
2-21	5.0	0.2	+ + +	Y	0.0	0.0	100.0	+
2-22	5.0	0.5	+ + +	Y	0.0	0.0	96.7	+
2-23	5.0	1.0	+ + +	Y	0.0	0.0	100.0	+
2-24	5.0	2.0	+ + +	Y	0.0	0.0	100.0	+
2-25	5.0	5.0	+ + +	Y	0.0	0.0	100.0	+

See Table 1 for footnotes.

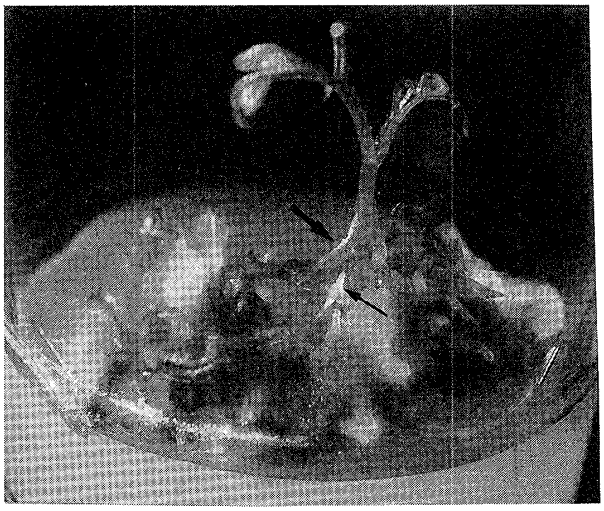

Fig. 1a. Plant regeneration from hypocotyl-derived calli of African marigold (*Tagetes erecta* L.). Thick and thin arrows indicate shoot and root, respectively.

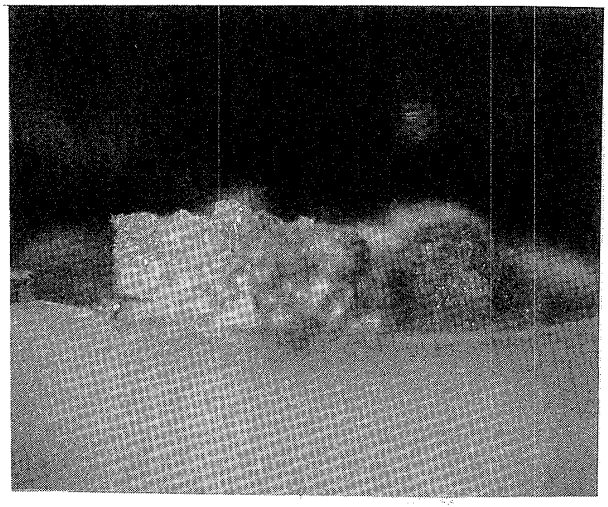

Fig. 1b. Leaf-derived calli from African marigold (*Tagetes erecta* L.)

transfer of calli onto fresh culture medium proved to be an effective method of enhancing callus formation and plant regeneration in marigold. The results also indicate that the growth and metabolism of calli from African marigold markedly depended on the concentration of plant growth regulators and the organ-specificity. Adequate combinations of NAA and BA in the culture medium induced calli and subsequently plant regeneration in hypocotyl explants, whereas only roots from calli of leaf explants. Although it is preferable to use hypocotyl explant for plant regeneration studies, the leaf seems to be a suitable organ for callus formation (Fig. 1b) as reported by KETEL (1987). However, the difficulty of regenerating plantlets from leaf-derived calli needs further investigation.

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マリーゴールドにおけるカルス誘導と植物体分化

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マリーゴールドは，殺線虫効果を有する植物である．本研究で使用した品種（African Tall）は，殺線虫専用種として使用されている．本研究は，マリーゴールドの殺線虫成分（thiophene）に関与する遺伝子を重要作物に導入するための前段階として，マリーゴールドの培養系の確立を検討した．

材料として，マリーゴールドの胚軸および葉片を用いた．胚軸および葉片を異なる濃度のBAおよびNAAの組み合わせを含むMS培地に置床した．胚軸由来カルスは，置床後褐変が激しく，そのため生育の停滞，枯死が起こったため，置床に先だって切りとった胚軸を1％アスコルビン酸で処理した．さらに，カルスを再分化培地に置床するに先立って一週間ごとに3回新鮮培地に移し換えた．この方法は，カンショなどの培養の場合も有効であるが，マリーゴールドの場合は予備的に行った培地への活性炭添加よりも有効であった．

胚軸由来カルスでは，0.2～1.0 mg/l NAAおよび0.5～5.0 mg/l BAを含む培地で植物体再分化が見られた．しかし，葉片由来カルスではどの培地でも不定根形成のみ観察された．ただし，いくつかの培地でグリーン・スポット形成が見られた．さらに，葉片由来カルスでは，5.0 mg/l NAAおよび0.2～5.0 mg/l BAを含む培地で黄色の大きなカルスが形成された．しかし，今後これら葉片由来のカルスを有効利用するには，植物体再分化の問題を解決する必要がある．