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— SHORT COMMUNICATION —

Agrobacterium rhizogenes mediated hairy root induction in *Oxystelma esculentum* (L.f) R.Br.

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This report describes a technique to induce hairy roots in *Oxystelma esculentum* (L.f) R.Br. Explants of hypocotyls and cotyledons derived from *in vitro* germinated seedlings were aseptically co-cultivated with *Agrobacterium rhizogenes* strain 15834 and cultured in half- and full-strength MS and B_5 basic media. The infected hypocotyls responded well and genetic transformation was observed with reference to the total biomass and number of hairy roots. Transgenic plants often exhibited altered phenotypes compared with controls. The response of hairy root induction in hypocotyls was found to be 96%, the mean biomass 83.8 ± 0.48 g and the mean number of hairy roots 35.6 ± 0.97. The hairy root syndrome is displaced by plants regenerated from Ri-transformed roots. Combined expression of the *rol* (A, B and C) loci of *A. rhizogenes* was observed and Ri-plasmid was involved in the establishment of the hairy root syndrome.

Key words: *Agrobacterium rhizogenes*, Ri-tDNA, *Oxystelma esculentum*, transgenic plants, MS and B_5 media.

INTRODUCTION

Plant cell cultures have a tendency to become genetically and biochemically unstable and often synthesize low levels of useful secondary metabolites. However, induced hairy roots using *Agrobacterium rhizogenes* have been explored and intensively utilized for a high production of secondary metabolites (Merkli *et al*., 1997; Kittipongpatana *et al*., 1998).

The soil-born plant pathogen *A. rhizogenes* is responsible for adventitious (hairy) root formation upon agroinfection and accumulation of biochemicals in plant metabolism (Grant *et al*., 1991). Root induction is due to stable integration of the Ri-tDNA (transferred DNA) into the plant genome and its subsequent expression (Chilton *et al*., 1982). Roots can then be cultured on hormone-free media. Hairy root cultures have three main advantages: genetic stability, cultivation without any addition of growth regulators, and ability to give high final biomasses from low inocula. These transformed root cultures are considered as a promising source for the production of biologically active metabolites (Toivonen *et al*., 1989, 1992; Saito *et al*., 1992; Toivonen, 1993; Bourgaud *et al*., 1997). Optimization of culture conditions, selection of highly productive strains, and addition of precursors are the most common approaches to maximize the yield of metabolites (Misawa, 1994).

Many important secondary metabolites are synthesized in root tissues and are stored *in situ* or transported to other organs (Waller & Nowacki, 1978). A vast amount of literature is available on the production of high volume phytochemicals such as alkaloids (Toivonen *et al*., 1991) and terpenoids (Weathers *et al*., 1997) by *Agrobacterium*-transformed hairy root cultures. To our knowledge, there are no media specifically formulated for hairy root cultures, hence the most commonly used media are the MS (Murashige & Skoog, 1962) and the Gamborg's B_5 (Gamborg *et al*., 1968). These are composed of macro- and micronutrients, vitamins and sucrose, needed to optimize hairy root growth and secondary metabolite production. The roots and leaves of *Oxystelma esculentum* are reported to possess antiseptic, depurative, and

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galactagogue substances. The fresh roots are prescribed as jaundice (Chadha, 1966). Because of the presence of valuable phytochemicals in the root of *O. esculentum* and the importance of this plant in the treatment of jaundice, a study was initiated for the *Agrobacterium*-mediated hairy root induction.

MATERIALS AND METHODS

Plant material

Mature seeds of *O. esculentum* were collected from the riverbank of the Tiruchirappalli District (southern India). The seeds were thoroughly washed in tap water for 15 min and immersed in 70% ethanol for 1 min. They were then washed with an aqueous solution of 5% Teepol for 3 min, and 1% Bavistin fungicide for 3 min and surface-sterilized in 1% $HgCl_2$ for 5 min, followed by five rinses in sterile distilled water. Seeds were germinated on hormone-free MS (Murashige & Skoog, 1962) and B_5 (Gamborg *et al*., 1968) media at 25 ± 2°C with a photoperiod of 16 h light and 8 h dark, using 40 μmol m^{-1} s^{-1} cool white fluorescent tubes. Cotyledon and hypocotyl segments (approximately 1 cm long) of 3-week-old aseptically grown seedlings were used as a source of explants for genetic transformation.

Activation of bacterial strains

Agrobacterium rhizogenes wild type strain ATCC 15834 stored at 4°C was transferred to a nutrient agar solidified medium and cultured at 25°C in the dark. A single bacterial colony was inoculated in 10 ml of liquid nutrient broth medium and cultured at 25°C for 24 h in the dark on a shaker agitated at 120 rpm. The bacterial suspension was adjusted to 0.6-0.8 OD at 600 nm.

Initiation and establishment of hairy root culture

Excised cotyledon and hypocotyl segments (approx. 0.5-1 cm in length) from 5-day-old seedlings of *O. esculentum* were used as explants for co-cultivation with *A. rhizogenes*. A wound was gently made in the explants with a sterilized surgical blade. For co-cultivation, wounded segments of cotyledons and hypocotyls were immersed in the bacterial broth culture of 0.6-0.8 OD at 600 nm. For 15-20 min, the explants were blotted on to remove any excess of bacterial inoculum. All explants were co-cultivated on Petri plates containing MS and B_5 hormone-free (basic) agar media and incubated in the dark for 2 days. After co-cultivation, the explants were removed and washed twice with sterile distilled water. They were then blot-dried with a sterile Whatman No-1 filter paper and transferred to a fresh MS and B_5 hormone-free agar medium containing 300 mg l^{-1} cefotoxime (Alembic chemical company, India). Bacteria were eliminated from the hairy roots by transferring the explants to a fresh medium containing cefotoxime at intervals of 7-10 days (3-5 times). The bacteria-free roots were subcultured on full- or half-strength MS and B_5 basal liquid media, and incubated on an orbital shaker (120 rpm) under dim fluorescent light at 25 ± 2°C (subculturing every 15 days). The response of the different explants for induction was recorded.

Effects of MS and B5 media

Hairy root cultures of *O. esculentum* were grown separately in MS (containing 100 mg l^{-1} inositol, 3% sucrose, pH 5.5) and B_5 (containing 100 mg l^{-1} inositol, 2% sucrose, pH 5.7) media. The mass of hairy root culture was inoculated at 25 ± 2°C, with 40 μmol m^{-1} s^{-1} cool white fluorescent tubes and 16 h photoperiod.

Statistical analysis

Experiments were set up in a Randomized Block Design and were repeated three times. Ten to fifteen explants were used per treatment in each replication. Observations were recorded with reference to the percentage of response, total biomass of hairy roots, and number of hairy roots, respectively. The treatment means were compared using the Duncan's multiple rang test at a 5% probability level, according to Gomez & Gomez (1976).

RESULTS

The transformation of *O. esculentum* was carried out using *A. rhizogenes* strain ATCC 15834 in MS and B_5 media. Hairy root formation was observed after 7-10 days of cotyledon and hypocotyl explant infection without callus formation (Fig. 1), while all control explants (uninfected cotyledons and hypocotyl explants) produced callus only after 7-10 days of inoculation. In this study, percentage of response, total biomass in fresh weight, and number of produced hairy roots were observed in experimental and control series.

Hairy root induction from hypocotyl explants

High survival rates were observed in infected hypocotyl explants (Table 1). This could be due to the hy-

TABLE 1. Frequency of hairy roots from *Oxystelma esculentum* hypocotyl explants induced by *Agrobacterium* strain ATCC 15834 on MS and B_5 media

Medium concentration	Control (uninfected explants)			Infected (hypocotyls)		
	% of response	Biomass fresh weight (g)	No. of hairy roots	% of response	Biomass fresh weight (g)	No. of hairy roots
½ MS	–	–	–	80%	56.3 ± 0.54^{bc}	26.0 ± 0.81^{bc}
MS	–	–	–	96%	83.8 ± 0.48^{a}	35.6 ± 0.97^{a}
½ B_5	–	–	–	76%	52.3 ± 0.54^{cd}	22.0 ± 1.21^{cd}
B_5	–	–	–	78%	60.6 ± 0.97^{b}	27.0 ± 0.46^{b}

The means and standard errors are presented for each column. Means sharing at least one letter are not significantly different at the 5% level (Duncan's multiple range test)

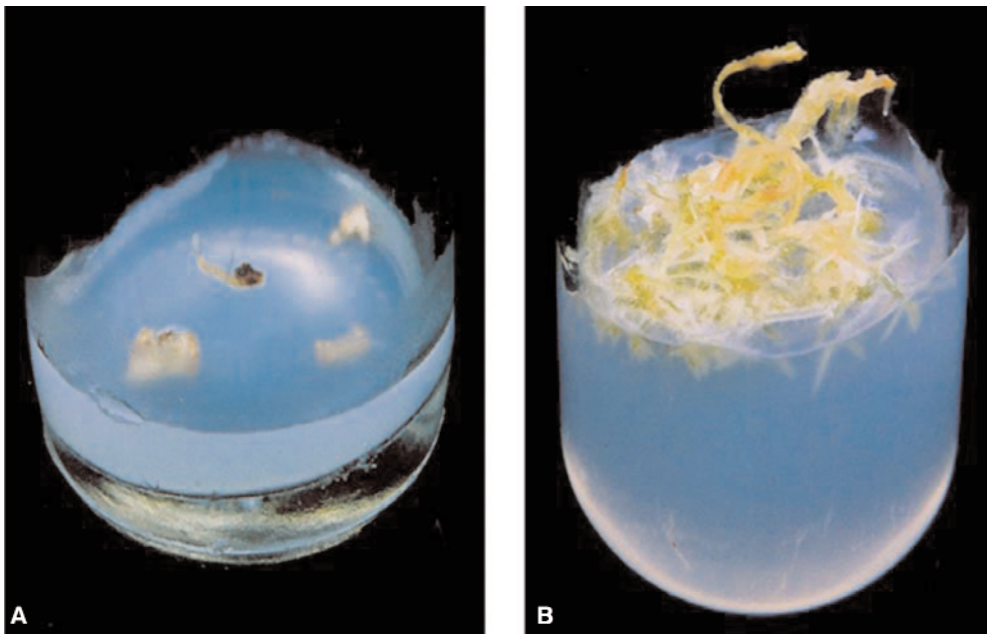

FIG. 1. Hairy root induction from hypocotyl explants of *Oxystelma esculentum* infected with *Agrobacterium rhizogenes* (ATCC 15834) on a full-strength MS medium. A: 30 days old uninfected hypocotyl explants (control); B: 30 days old hairy roots from infected hypocotyl explants.

pocotyl segments being cultured in half- and full-strength MS and B_5 media. Rooting percentage was significantly influenced by co-cultivation with ATCC 15834 strain inoculated in a full-strength MS basic medium. Root number increased in this medium (Table 1). The hypocotyl segment co-cultivated with strain ATCC 15834 produced a significantly greater rooting percentage and a mean number of hairy roots compared with the uninfected explants (Table 1). However, uninfected hypocotyl segments did not produce any hairy roots. The full-strength MS medium is suitable, when compared with the full-strength B_5 and the half-strength MS and B_5 media.

TABLE 2. Frequency of hairy roots from *Oxystelma esculentum* cotyledon explants induced by *Agrobacterium* strain ATCC 15834 on MS and B_5 media

Medium concentration	Control (uninfected explants)			Infected (hypocotyls)		
	% of response	Biomass fresh weight (g)	No. of hairy roots	% of response	Biomass fresh weight (g)	No. of hairy roots
½ MS	–	–	–	80%	53.6 ± 0.36^{bc}	24.6 ± 0.71^{bc}
MS	–	–	–	95%	81.2 ± 0.71^{a}	34.3 ± 0.97^{a}
½ B_5	–	–	–	72%	51.5 ± 0.62^{cd}	21.0 ± 0.81^{cd}
B_5	–	–	–	79%	59.6 ± 0.27^{b}	25.4 ± 0.27^{b}

The means and standard errors are presented for each column. Means sharing at least one letter are not significantly different at the 5% level (Duncan's multiple range test)

Hairy root induction from cotyledon explants

The cotyledon explants were co-cultivated with *A. rhizogenes* strain ATCC 15834 and cultured in full- and half-strength MS and B_5 media. The best result was obtained in full-strength MS medium which gave 95% of hairy root induction (Table 2) and also a rich biomass and a high total number of hairy roots. The infected cotyledon showed hairy root production compared with the control, where there was no response of hairy root induction in the cultured cotyledon, uninfected with *A. rhizogenes* (Table 2). However, the hypocotyl explants produced a high amount of biomass and also secondary metabolites compared with the cotyledon root culture. The hypocotyl explants revealed a better hairy root induction than those in full-strength MS basic medium.

DISCUSSION

Secondary metabolites have been reported to be produced in cell cultures in many plants. Very often they are produced in low concentrations by undifferentiated plant cell cultures, probably because growth without differentiation is incompatible with the expression of secondary metabolic pathways. An alternative approach to the production of plant secondary metabolites in cell cultures is the use of organized or differentiated cultures, especially transformed (hairy) root cultures. There is some evidence that transformed roots can produce numerous secondary metabolites (Yonemitsu *et al*., 1990; Granicher *et al*., 1992) when compared with the whole plant production. Furthermore, they show a long-term stable production of secondary metabolites (Maldonado-Mendoza *et al*., 1993) and generally grow faster than the untransformed roots or cell cultures.

Natural transformation by exogenous DNA is documented in bacteria (Romanowski *et al*., 1993; Lorenz & Wackernagel, 1994). DNA transfer from bacteria to higher organisms occurs in the laboratory (Tepfer, 1983; Heinemann & Sprague, 1989). Among the probable cases of "horizontal" gene transfer, interaction between the soil bacterium *A. rhizogenes* and higher plants provide arguments for the importance of gene transfer from bacteria to plants.

Soil-born pathogens of the genus *Agrobacterium* are able to transfer a part of their DNA, (T-DNA carried on a large plasmid) to the genome of a host plant cell. *Agrobacterium rhizogenes* is the causal agent of "hairy root" disease in plants and has been used for the production of hairy root cultures from a multitude of species. Transformed roots are able to synthesize secondary products at a higher rate than wild type roots. An efficient *A. rhizogenes*-mediated protocol has been developed for the establishment of transgenic *O. esculentum* hairy root cultures. Insertion of Ri T-DNA is known to affect plant phenotype, including reduced height and increased rooting ability (Tepfer, 1984, 1989).

The results here show that different experimental protocols could significantly affect the survival percentages and the subsequent rooting percentages and root number. For instance, survival and rooting were lower in controls (uninfected explants). Moderate and low survival rates were recorded in half-strength MS and B_5 media. Interestingly, when hypocotyl segments inoculated with ATCC 15834 were cultured in a full-strength MS medium, a significantly higher rooting percentage and root number were observed (Table 1) without any addition of auxins.

Several other studies have also reported the differential efficiency of *A. rhizogenes* strains in promoting the formation and growth of hairy roots, in addition to their variable ability to induce hairy root deve-

lopment. Different *A. rhizogenes* strains also affected growth rate, saponin production and varying ratio of astragalosides in transgenic root cultures of *Astragalus mongholicus* Bge. (Ionkova *et al*., 1997). Strain 15834 of *Agrobacterium* was among the most effective in promoting hairy root growth and saponin synthesis. This strain also influenced development, growth rate and hyoscyamine production in transformed root cultures of *Hyoscyamus muticus* (Vanhala *et al*., 1995). C58 C1 was among the most virulent strains, which resulted in root cultures with the highest alkaloid content. By contrast, strain 15834 was the least effective for the induction of hairy roots of *H. muticus*. Strain 15834 was also most virulent and efficient for hairy root development in *Catharanthus roseus* G. Don. (Brillanceau *et al*., 1989). The selection of an effective *Agrobacterium* strain for the production of a transformed root culture is highly dependent on the plant species, and must be determined empirically. The differences in virulence, morphology and growth rate are partially related to the variety of plasmids contained within each bacterial strain.

The cultured hypocotyl segments described here are a good system for the study of hairy root induction in *O. esculentum*. Since the process of root formation can be easily manipulated under highly defined experimental conditions, and rooting response could be relatively high, this makes the system attractive for further investigation. It will pave the way for the commercial exploitation of the bioactive components present in the plant.

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