OPTIMISING PRODUCTION OF CARROT HAIRY ROOTS

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ABSTRACT

This research describes the production of carrot hairy roots for monoxenic culture of New Zealand arbuscular mycorrhizal fungi (AMF). Induction of hairy roots on mature carrot root sections was carried out using *Agrobacterium rhizogenes* isolate A4T that had been grown in either Lauria-Bertani (+/- acetosyringone) or Yeast Mannitol medium (24 or 48 h incubation). Results showed both methods could initiate hairy root production. The source of the carrots was one of the most important factors, with mature, freshly harvested carrots showing better hairy root production compared to cool-stored carrots. Dissection of the cortex to expose the cambium had a positive effect on the least optimal treatment.
Keywords: vesicular arbuscular mycorrhiza, monoxenic, carrot hairy root, *Agrobacterium rhizogenes*.

INTRODUCTION

Arbuscular mycorrhizal fungi (AMF) are inherently challenging to work with as they cannot be cultured outside their host plants and are difficult to locate and separate from surrounding root tissue. In 1975, Mosse & Hepper developed the first simple *in vitro* systems for culturing AMF and these crude systems have since been refined to current monoxenic, hydroponic and aeroponic systems. The monoxenic method involves growing sterile AMF spores on dual culture plates with transformed carrot (*Daucus carota*) roots (St-Arnaud et al. 1996). The carrot roots are transformed by a wildtype strain of *Agrobacterium rhizogenes* containing a plasmid that induces a hairy root phenotype. These adventitious roots can then be cultured *in vitro* in medium devoid of plant hormones, where they grow very rapidly with a characteristic highly branched and non-geotropic pattern (David et al. 1984). The combination of transformed carrot roots and sterile AMF spores can be used to produce "dual *in vitro* cultures" that provide an efficient method of producing abundant (typically over 5000) spores and mycelia in a 9 cm Petri plate (Becard & Fortin 1988; Diop et al. 1992). This system also allows the cold storage (4˚C) and subculture of AMF by the re-association of mycorrhizal transformed root pieces with another carrot root on a new agar medium (Plenchette et al. 1996). This method of artificial culture is a valuable tool for the study of arbuscular mycorrhizal fungi as it avoids the interactions of other inhabitants of the rhizosphere, which may cause confusion.

Although carrot hairy roots have been used in monoxenic culture of AMF since 1988, there are no reports of an optimised method for their production. The objective of this work was to compare two standard methods and modifications to those methods for hairy root production in carrots. In addition, three sources of carrots, mature cool-stored, mature fresh and spring carrots, were compared as substrates for hairy root initiation.

MATERIALS AND METHODS

Bacterial strain propagation

Agrobacterium rhizogenes isolate A4T was provided by Crop & Food Research, Lincoln. A suspension of this strain was prepared by inoculating a loopful of the colony into Lauria-Bertani (LB) broth (10 g/litre Bacto tryptone, 5 g/litre Bacto yeast-extract,

5 g/litre NaCl) and incubating overnight on a shaker at 28˚C at 150 rpm (Becard & Fortin 1988). This liquid stock culture was used for further subcultures in LB and Yeast Mannitol (YM) (2 g/litre mannitol, 3 g/litre glucose, 5 g/litre sucrose, 0.5 g/litre K_2HPO_4, 0.2 g/litre $MgSO_4.7H_2O$, 0.1 g/litre NaCl, 0.05 g/litre $CaSO_4.2H_2O$, 0.1 g/litre NH_4Cl, 1.0 g/litre yeast extract) with and without acetosyringone (50 mM).

Preparation of carrot roots

Two sources of carrots (cultivar unknown), both purchased in October, were used in these experiments. Source 1 was from a local supermarket and had been kept in cold storage. Source 2 was from a local market gardener and had been freshly harvested. Carrots were washed, peeled and surface sterilised in 1% sodium hypochlorite for 15 min with occasional stirring (Becard & Fortin 1988). They were further rinsed three times (5 min each) in sterile distilled water. Prior to cutting into discs they were dipped in ethanol and flamed. Each carrot was sliced into several 0.5 cm thick discs and the cortex from approximately half of the pieces was further trimmed to expose the cambium.

Carrot disc inoculations

Two standard methods and modifications to these methods were compared for carrot hairy root production. The first method, described by Smith & Dickson (1997), was a modification of an earlier method by Becard & Fortin (1998). For each carrot source, the shoot side of the carrot discs were dipped into 48 h liquid cultures of *A. rhizogenes* A4T in YM medium for 10 s. The modified treatment used a reduced incubation time (24 h) to try and improve transformation efficiency. The second method, described by Christey & Braun (2004), used carrot discs dipped in a 4 h liquid culture of *A. rhizogenes* A4T in LB medium for 10 s. The modified treatment included acetosyringone in the culture medium in an attempt to improve transformation efficiency. Controls consisted of carrot discs dipped into uninoculated YM and LB media. Treated discs were removed from the culture medium, dried on sterile filter paper and then placed onto 6% water agar with the inoculated sides facing up and incubated at 28˚C for 2-3 weeks. For each treatment, the percentage carrot discs producing hairy roots was calculated and a Generalised Linear Model with binomial distribution was used to determine significant differences between carrot sources.

Maintenance of hairy root cultures

Proliferating discs were transferred to individual pottles containing 50 ml of Modified White's (MW) medium (731 mg/litre $MgSO_4.7H_2O$, 453 mg/litre $NaSO_4.10H_2O$, 80 mg/litre KNO_3, 65 mg/litre KCl, 21.5 mg/litre NaH_2PO_4, 288 mg/litre $Ca(NO_3).4H_2O$, 30 g/litre sucrose, 8 mg/litre NaFeEDTA, 0.75 mg/litre KI, 6 mg/litre $MnCl_2.4H_2O$, 2.65 mg/litre $ZnSO_4.7H_2O$, 1.5 mg/litre H_3BO_3, 0.13 mg/litre $CuSO_4.5H_2O$, 0.0024 mg/litre $Na_2MoO_4.2H_2O$, 3 mg/litre glycine, 0.1 mg/litre thiamine hydrochloride, 0.1 mg/litre pyridoxine hydrochloride, 0.5 mg/litre nicotinic acid, 50 mg/litre myoinositol, 0.4% Phytagel [Sigma]) as described by Becard & Fortin (1988). The cultures were inverted onto antibiotic amended medium (300 mg/litre of timentin). Three successive subcultures were carried out on this medium to eliminate bacteria from the transformed roots. Once free of bacteria, clonal cultures were initiated by aseptically excising 1 cm long root apices and transferring them into fresh pottles containing MW medium. Carrot hairy roots for dual culture were routinely maintained on MW medium indefinitely.

RESULTS

The number of pieces for each treatment, and the percentage production of hairy roots at the end of the 6 week growth period are shown in Table 1. Two kinds of roots started forming on the carrot root discs after 10 days. The first were "hairy roots"; these were delicate, aerial and much branched. In contrast, the second type were "non- hairy" roots; these were slightly thicker, unbranched and exhibited strong negative geotropism. Roots were initiated from both sides of the discs. Hairy root initiation continued to occur from 10 days (earliest) to 3–4 weeks. Approximately 80% of controls discs for carrot source 1 and carrot source 2 were still healthy after 6 weeks but ultimately began to rot. Two types of rot were observed. The first was due to overgrowth of the *A. rhizogenes*

and had a characteristic slimy appearance under which the carrot discs remained intact. The second type of rot was a general browning and collapse of the carrot disc.

TABLE 1: The number of carrot discs producing hairy roots, no roots or going rotten. Carrot discs were inoculated with different treatments of *Agrobacterium rhizogenes* (A4T) with or without the cortex exposed.

			Number of carrot discs					
				Hairy roots				
Carrot Source	Treatment	Inoculation	Total	+ Cortex	- Cortex	No roots	Rotten	% Hairy roots
1	1	YM Control	11	0/6	0/5	7	4	0
1	2	48h YM	20	0/14	0/6	0	20	0
1	3	24h YM	14	1/7	0/7	9	4	7
1	4	LB Control	14	0/9	0/5	13	1	0
1	5	4h LB	25	0/14	4/11	21	0	16
1	6	4h LB + aceto.	22	3/12	3/10	0	16	27
2	7	YM Control	8	0/4	0/4	8	0	0
2	8	48h YM	39	3/17	13/22	21	2	41
2	9	24h YM	30	8/15	8/15	1	13	53
2	10	LB Control	12	0/4	0/8	8	4	0
2	11	4h LB	27	2/10	6/17	19	0	30
2	12	4h LB + aceto.	20	3/9	3/11	0	14	30

The best hairy root production for carrot source 1 was treatment 6 (LB + acetosyringone) and treatment 9 for carrot source 2 (24 h YM). Freshly harvested carrots (source 2) produced a consistently higher % of roots in comparison to the cold stored supermarket carrots (source 1). There was a significant difference (P=0.01) between carrot sources for the YM treatments (2-3, 8-9), but not for the LB treatments (5-6,11-12). Trimming the discs to remove the cortex and expose the cambium resulted in significantly (P=0.01) improved hairy root formation in treatment 8 (Table 1). The clonal cultures generated by subcultured root apices that had undergone three successive generations on antibiotic (timentin) containing medium were maintained indefinitely on antibiotic free medium.

DISCUSSION

Carrots are one of the most susceptible species of plant for hairy root production (Christey & Braun 2004). It is, therefore, not surprising that most treatments resulted in hairy root formation, although not equally so. Although carrot hairy roots have been used to initiate monoxenic AMF culture since 1988, optimisation of methods for their production have not been described. In this study, two established methods of carrot hairy root initiation were modified in an attempt to optimise hairy root initiation in terms of the number of root initiations and the health (lack of rotting) of the explants. In addition, the effect of carrot source on the outcome of these methods was assessed. Studies have shown that acetosyringone promotes transformation by actively inducing the transfer of T-DNA from *Agrobacterium* to the plant (Kyung-Hwan et al. 1996). For both carrot sources, the addition of acetosyringone to the method of Christey & Braun (2004) did not result in an increase in transformation efficiency. Reducing the YM incubation time from 48 to 24 h also did not improve transformation efficiency.

Irrespective of treatment, there was a consistent difference between the two carrot sources, with the fresh carrots (source 2) from the market garden invariably better at

initiating hairy roots overall. This may be due to either the less dormant nature of the carrots or to cultivar differences between the fresh and cool stored carrots. In additional work (data not shown), spring carrots were also trialed as a substrate for hairy root initiation. Whilst almost all of the discs from those carrots rapidly initiated roots, they also rotted very quickly and this led to the experiment being abandoned. The majority of the rot in this case was due to *A. rhizogenes* overgrowth. This observation supports the premise that less dormant carrots are quicker to produce hairy roots. However, cultivar differences cannot be discounted and have been reported as a factor influencing hairy root initiation (Christie & Braun 2004).

It was suggested by Smith & Dickson (1997) that trimming the carrot discs to expose the cambium would improve the generation of healthy hairy carrot roots. These results showed that exposure of the cambium only affected carrot source 2 (market garden) in the 48 h YM treatment (12). This treatment was considered to be the least optimal as the 48 h YM culture had low levels of active bacteria in culture due to nutrient depletion. It is probable that in this suboptimal treatment, exposing the meristematic cambium tissue facilitated the few transformation events that occurred. However, this method was not consistently effective and did not improve hairy root initiation of the cool stored carrots (source 1).

In summary, the best method for initiating hairy root production in carrots was the 24 h YM medium in combination with a fresh source of mature carrots. The use of this method to produce monoxenic cultures of New Zealand species of AMF will provide a valuable tool for the description and recording of the hyphal morphology and branching, sporulation dynamics and spore ontogeny of indigenous strains.

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