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INFLUENCE OF MEDIA GELLING AGENTS ON ROOT BIOMASS AND *IN VITRO* VA-MYCORRffIZAL SYMBIOSIS OF CARROT WITH *GIGASPORA MARGARITA*

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ABSTRACT

An *in vitro* study with Ri-TDNA transformed roots of carrot *(Daucus carota)* was carried out to evaluate the role of macro-elements contributed as impurities in the gelling agent (phytagel) over and above those present in the minimal (M) medium. Production of root biomass was taken as a measure to quantify the influence of macro-elements added to the minimal medium. The levels of phosphorus when adjusted to 1.19 mg/1 and 1.09 mg/l, lead to dry root biomass production at par with the control.

Attempts made to lower the amount of impurities in phytagel by de-ionization using different alkalies, proved NaOH to give the best results in terms of relatively high amount of root biomass. In an in *vitro* dual culture system with carrot as host and *Gigaspora margarita* as the vesicular-arbuscular mycorrhizal fungus, phytagel impurities helped to produce maximum number of infection units and auxiliary cells when phytagel was added to the minimal medium.

Key words: *Agrobacterium rhiiogenesfDaucus* caro/a/Gelling agents/diaspora *margarita/Macro*-elements/Vesicular-arbuscular mycorrhiza/Transformed roots.

INTRODUCTION

Production of VAM fungi under axenic conditions continues to be one of the most challenging goals of modem biology (Becard and Piche 1992). Mosse and Hepper (1975) were the first to report a simplified *in vitro* system for the study of VAM development wherein they used excised roots instead of whole plants. Mugnier and Mosse (1987) modified the technique further by using Ri-TDNA transformed hairy roots as the host tissue. Plants with such tumoral roots are potentialy valuable for this kind of study as they not only have exceptionally low nutritional requirements but also allow cultivation on synthetic media axenically (Nuutila *et al.* 1995).

Amending an appropriate medium for co-cultivating the two components, plant roots and the fungus are the most important factor for a successful vesicular-arbuscular mycorrhizal formation in root organ culture (Becard and Fortin 1988). After the first

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report about the M medium (Becard and Fortin 1988), the agar component in this media was replaced by other gelling agents, such as gellen gum (Gel Gro, ICN Biochemicals, USA) or phytagel (Sigma Chemical Co., USA). These gelling agents made the media more transparent, which facilitated observation under the light microscope.

Diop *et al.* (1992) reported that these gelling agents are polysaccharides with very few impurities. However when analyzed, they were found to contain a number of elemental ions, which significantly increased the concentration of many major and minor ions *in* the medium. Taking these observations into account, the present study was undertaken to optimize the level of some of the major elements, which are known to help in root growth and eventually improve *in vitro* culture conditions towards a more stronger symbiosis hi the dual culture system.

MATERIALS AND METHODS

Transformation of carrot *(Daucus carota* L.)

Agrobacterium rhizogenes strain (A4 wild type ATCC 43057) was used to induce transformation (Mugnier and Mosse 1987). A loopfull of overnight grown culture was suspended in MS broth (Murashige and Skoog 1962) (optical density adjusted between 0.6 and 0.7). The tap root of carrot was obtained from agricultural fields and was surface sterilized for 10 min. in 30% HaOi solution and rinsed three tunes hi sterile, distilled water. The cut ends of roots were then dipped in bacterial suspension for 15 min., filter soaked and put on MS medium supplemented with 500 mg/1 cefotaxime. After about 3-4 weeks, roots emerging from the cut ends were excised and put on MW medium (Becard and Fortin 1988). Healthy white root tips with numerous laterals showing negative geotropism (unlike the non-transformed root cultures) were routinely maintained on M 0.2% medium (w/v) (Becard and Fortin 1988).

Transformation of cucumber *(Cucumis sativus* L.)

Cucumber seeds were surface sterilized as described above and germinated in the dark at 27°C on moist, sterile filter paper. When radicles were approximately 2 cm long, the seedlings were transferred to MS medium slants and kept in growth room at 25°C with 16/8 h light/dark photoperiod and a relative humidity of 65±5%. Once the plants became healthy, sections and slanting incisions were made on stems and leaves. Cut ends were then subjected to transformation. The roots that emerged (showing negative geotropism) were subsequently maintained on M 0.2% medium (w/v).

Non-transformed root cultures

Seeds of tomato *(Lycopersicon esculentum* Mill.), were surface sterilized and the seedlings were put on MS slants. After establishment, healthy roots were excised and placed on MW medium. After 3-4 weeks, they were transferred onto M 0.2% medium (w/v) and maintained subsequently on the same medium.

Testing host for colonization

Fungal inoculum

Azygospores of VAM fungus, *Gigaspora margarita* Becker and Hall (DAOM 194757, deposited at the Biosystematic Research Center, Ottawa, Canada), grown in pots in a greenhouse, were collected by wet sieving (Gerdmann and Nicolson 1963) and purified by density gradient centrifugation in 60% urografm (Furlan *et al.* 1980). Spores thus obtained were then surface sterilized with 2% chloramine T and rinsed in antibiotic solution which consisted of 1% streptomycin and 0.5% gentamycin (Becard and Fortin 1988). They were then stored on 1 % sterile water agar at 4°C. Viability of the spores was ascertained visually by their light colour and globule filling. Only viable candidates were picked up and inserted into 1 % sterile water agar and the plates were incubated in a CO_2 incubator (Biocenter 2001, Salvis, Switzerland) with 2% CO_2 at 26°C. Later on, germinated spores were transferred to M medium prepared with 0.4% (w/v) phytagel.

Selection of host

For selection of a suitable host for subsequent studies, various root cultures of carrot, tomato, and cucumber were examined for their affinity to *in vitro* symbiosis with G. *margarita*. Many of these cultures were developed in our laboratory (except for ERRC-I which was obtained from G. Becard, ERRC, Philadephia. USA) and classified on the basis of rate of root growth, (Table 1) as A=Fast (root growth > 2 cm/day), B=Moderate (root growth 1-2 cm/day), and C=Slow (root growth < 1 cm/day). GP-I root culture proved to be one of the fastest growing and besides being Myc+ was selected for subsequent studies.

Table 1. Host species tested for colonization study

	Growth rate		
Species	**A**	**B**	**C**
Carrot	ERRC-I		
	GP-I		
	GP-II	GP-III	
Tomato	**Tom**		
Cucumber	CU-I	CU-II	cu - III

Establishment of dual culture

In vitro dual culture was initiated from a single spore of *G. margarita* and a single healthy root tip. Different root cultures of carrot, tomato and cucumber were used to establish the experimental unit. The petri dishes were kept for incubation in the dark at 26°C in order to facilitate the growth of the germ tube (Becard and Piche 1989). The experiment was terminated at the end of 4 weeks. The medium was dissolved and roots were recovered using 10 mM sodium citrate buffer, pH 6 (Doner and Becard 1991). Roots were carefully picked, cleared and stained (Phillips and Hay man 1970). Stained roots were mounted on glass slides and observed at x 200 magnification under compound microscope attached to video camera and image analyser system (Leica, Switzerland) loaded with Quantiment 500^{+} software (Leica, Cambridge, UK) having colour option. To get the number of infectious propagule per unit length of root, the number of entry points were divided by total root length.

Root growth and rate limiting factors

In order to identify the role of certain major nutrient elements such as nitrogen, phosphorus, calcium, magnesium, and sulphur, the basic M medium was modified in different ways (Table 2). The level of each ion was altered while keeping the levels of j other ions constant (Table 3). Both lower and higher concentrations than what is prescribed were tested to study the role of deficiency, alternatively excess availability of the ion selected. Root tip length of carrot GP-I at start of an experimental unit was 2.5 cm in various treatments. At the end of four weeks, the root system was harvested according to Doner and Becard (1991) and dry weight of the total root system was recorded. The experiment was laid in completely randomised block design and data! were subjected to the analysis of variance (ANOVA) and means were separated using Duncan's multiple range test ($P<0.01$).

Table 2. Elemental (ionic) composition of M medium

Elemental ions selected for the present study	Levels of elemental ions contained in the basic M medium recipe (mg/l)	Level of ions contributed by phytagel in the M medium (when added @ 0.4%) (mg/l)	Final elemental make up of the medium (for selected ions) (mg/l)
Nitrogen	45.86	Nil	45.86
Phosphorus	1.09	0.40	1.49
Calcium	48.87	24.80	73.67
Magnesium	72.15	9.20	81.35
Sulphur	95.56	1.44	97.00

Table 3. Levels of selected ions attained with the basic M medium

Levels of selected ions (higher and lower than the M medium)	Elemental ions varied in the composition (mg/l)				
	Nitrogen	Phosphorus	Calcium	Magnesium	Sulphur
Higher levels					
H2	50.86	-	83.67	96.35	107
H1	-	-	78.67	86.35	102
Lower levels					
L1	40.86	1.19	68.67	-	92
L2	35.86	1.09	63.67	-	-
L3	-	0.99	-	-	-
L4	-	0.89	-	-	-
L5	-	0.79	-	-	-

Phytagel de-ionization and root growth

Phytagel was de-ionized by stirring it in distilled and de-ionized water in an Erlenmeyer flask at 60°C (Doner and Douds 1995). The requisite amount of mixed bed resin, TMD-8 (Sigma Chemical Co., USA) was then added and the mixture was incubated on a shaker at 60°C for four hours. It was then filtered through a clean cheesecloth. To the resultant turbid filtrate 200 mM KOH solution was added till it became clear (pH>8.0). This clear filtrate was then poured into pre-chilled iso-propanol and the flakes were recovered by re-filtering it through a cheesecloth and were dried overnight in an oven at 50 ± 5°C. The dried de-ionized phytagel was then subjected to ICP-AES (inductiviry coupled plasma-atomic emission spectroscopy) analysis to determine the concentration of ions (Table 4) and to decide subsequently the extent of changes to be made in the ionic composition of elements selected for the present study.

Table 4. ICP-AES analysis of various gelling agents

Gelling Agent	P	S	Mg	Na	Al	Pb	K	Mn	Fe	Ca	N
	(mg/kg)										
Phytagel	1049	394.4	2549	6789	116.8	BDL[a]	17040	26.54	96.55	7255	BDL[a]
EP agar	37.37	3010	191.2	4150	86.07	BDL[a]	92.09	5.471	169.5	2635	BDL[a]
De-ionized phytagel	165.5	117.3	267.7	1850	30.43	BDL[a]	30398	2.133	49.35	965.7	BDL[a]

[a]BDL = Below detectable limit

Test of de-ionized phytagel with different alkalies was done with an aim to improve rooting. In the routine procedure of phytagel de-ionization 200 mM KOH is added. This addition increases the K content of the medium from 135.35 mg/1 to 188.14 mg/1. Since the K level goes even higher than the impurity level of ordinary phytagel, different alternative alkalies, namely 200 mM NaOH, 200 mM $NaHCO_3$, 200 mM Trizma and 5% NH_4OH, were all tested to raise the pH of the solution. Young, actively growing root tips of carrot (GP-I, 2.5 cm long) were placed in petri plates, sealed and incubated in the dark at 27°C. Subsequently, at the end of four weeks, roots were recovered by the method of Doner and Becard (1991) and were dried in a hot air oven at 55 ± 5°C till a constant weight was achieved.

Test of different gelling agents and root colonization

Because phytagel contains significant amount of elemental impurities (Table 4), various gelling agents namely extra pure (EP) agar 0.7% (w/v) (Hi Media, India), phytagel 0.4% (w/v), de-ionized phytagel 0.5% (w/v) and agarose 0.4% (w/v) were used to solidify the M medium. Attempts were made to keep the physical state identical by varying the amount of different gelling agents in the media. *In vitro* dual cultures were established on these medium using a single spore of *G. margarita* and carrot GP-I root tip. Auxiliary cells formed after 2 weeks were counted and the experiment was terminated at the end of 4 weeks. After recovering (Doner and Becard 1991) and staining the roots (Phillips and Hay man 1970), the total number of infection units formed under various treatments were counted.

RESULTS AND DISCUSSION

Amended recipes and root biomass

An evaluation in terms of dry root weight production (g) for various amended recipes of nitrogen (Fig. la) shows that when the basic M medium recipe was modified, there was a reduction in dry mean root weight of carrot. The percentage reduction over control was highest (50.82) in HI compared to remaining two recipies (L140.86 mg/1 and L235.86 mg/1). As none of the set concentrations performed at par with the control, it is likely that either the reduced levels of nitrogen fail to meet the biochemical and physiological demands of the roots or the disturbances in the NO_3^-:NH_4+-N ratio in the medium lowered the recovery of dry root biomass.

In contrast to the basic M medium recipe, amongst all the five lower levels of phosphorus (P) tested, L1 and L2 recipes were at par with the control while L3, *L4* and L5 recipes produced significantly lower root biomass (Fig. 1b). Of all the

nutrients, insufficiency of P affects VAM symbiosis the most (Habte and Aziz 1991). It is also well established that high P content is detrimental to VAM establishment and decreases mycorrhizal infection (Abbott and Robson 1977, 1979; Menge *et al.* 1978) and probably, reduces the uptake of some minor elements namely Zn, Fe, and Cu. The M recipe was therefore, modified to incorporate lower levels of P as, subsequently, there is a need to optimise the P concentration in the medium for achieving better colonization.

Similarly, out of the four amended recipes for calcium (Table 3) root growth deteriorated to a great extent with H1 and H2 recipes (Fig. 1c). Between the other two recipes (L1 and L2) a considerably higher dry root weight was obtained with L1 (percentage reduction over control being 39.96) and a remarkably lower recovery of root biomass was observed in case of L2. Calcium is known to increase the rigidity of plant cell walls by cross-linking protein components by the formation of calcium pectate. Ca^{++} however, could adversely affect the biochemical and physiological processes within the root tissues as calcium at higher concentrations tends to shorten lateral branching in roots (Hirrel 1981) resulting in the low recovery of root biomass and subsequently reducing the uptake of cations like Mg. Lower recovery at L1 and L2 levels could be because of insufficient calcium, which leads to the disorganization of the plasma and vacuolar cell membranes, resulting in increased permeability and subsequent loss of certain vital nutrient ions (Burstrom 1968; Simon 1978).

Amendments carried out in minimal (M) media recipe to achieve two of the higher levels of magnesium, namely H1 and H2 produced significantly lower values of dry root biomass (Fig. 1d) when compared with the control. Out of the three different recipes made by amending sulphur in the basic (M) recipe, H1 and LI recipes produced significantly lower root biomass and were at par while H2 recorded 77.05% reduction over control (Fig. le).

Therefore, the original levels of the two elements Mg (87.35 mg/1) and S (97 mg/1) (in M medium and those added as impurities through phytagel) are optimal to meet the metabolic requirements of young carrot roots *in vitro*. The reduction in root biomass at higher levels of magnesium may be due to the ionic interactions such as Ca^{++} and K^{+}, resulting in lower availability of this element.

Colonization of roots

Maximum infection units and auxiliary cells were produced on ordinary phytagel, followed by EP agar, perhaps because certain very important elements present in the phytagel added significantly towards better root growth and symbiosis. A minimum of one infection unit and three auxiliary cells were formed on the medium prepared with de-ionized phytagel, while agarose plates produced the least number of auxiliary cells and infection unit (Table 5).

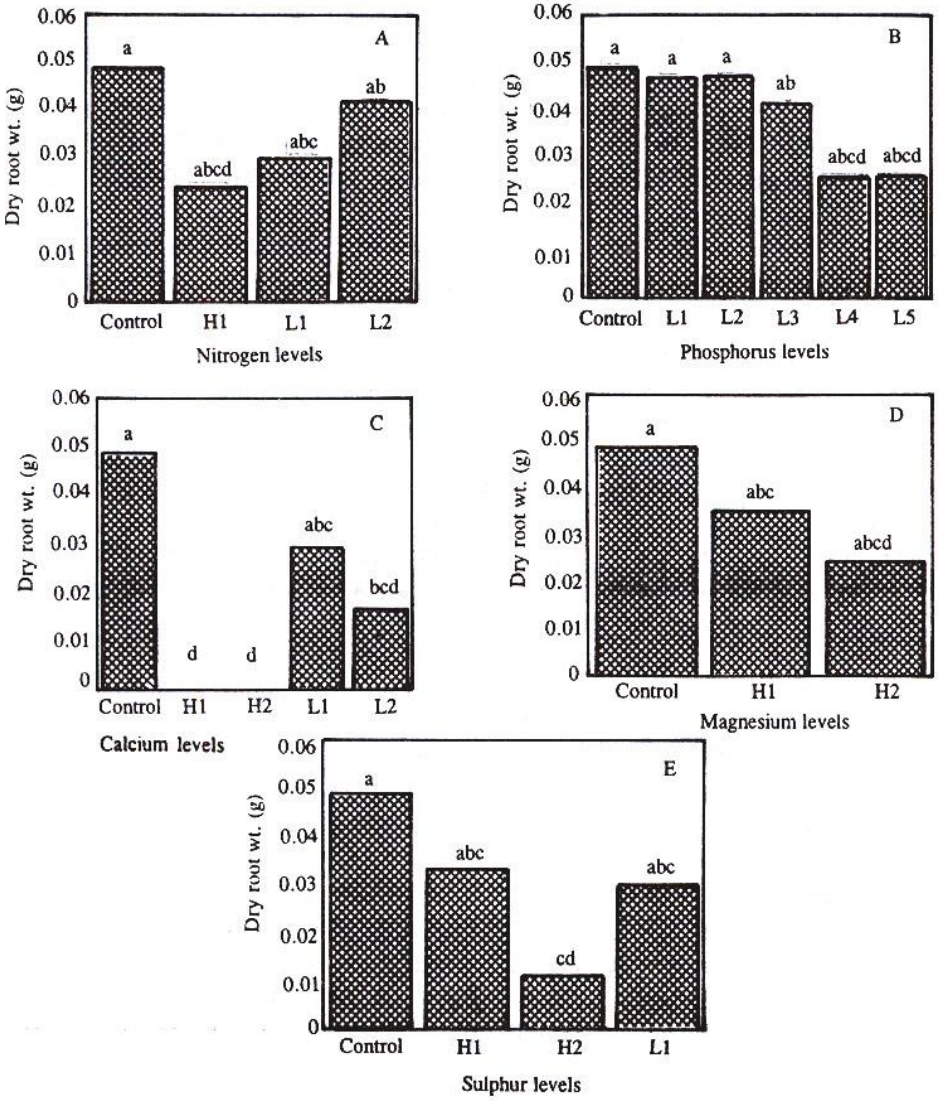

Fig 1. Effect of M medium recipes, amended for various nutrients, on *in vitro* root biomass. A-E, various recipes amended for nitrogen, phosphorus, calcium, magnesium and sulphur, respectively

Those bars followed by the same letter do not differ significantly by Duncan's multipke range test at $P<0.01$

Table 5. Test of various gelling agents for colonization of *Gigaspora margarita*

Gelling agents	No. of auxillary cells (after 2 weeks)	Infection units (after 4 weeks)
Extra pure agar (0.7%)	11	10ab
Agarose (0.4%)	1	0.67b
Phytagel (0.4%)	15	20.3a
De-ionized phytagel (0.5%)	3	1b

Within the same column, values (an average of 3 replicates) with a different letter are significantly different at $P < 0.01$ by analysis of variance.

ICP analysis of EP agar indicates very high sulphur and iron contents compared to phytagel. This could be detrimental to the root growth and colonization. In de-ionized phytagel the level of K rises significantly, affecting the symbiosis negatively. Agarose (0.4%) is devoid of impurities and its water-holding capacity is not as high as that of other gelling agents. Probably, due to this, root growth ceases after some time and affects drastically the colonization process.

Root biomass production in phytagel de-ionized with different alkalies

The dry root biomass values obtained with phytagel de-ionized by NaOH and Trizma were highly significant and were at par, while the remaining alkalies tested for de-ionization produced significantly lower root biomass values (Fig. 2). Though sodium is one of the critical components of the medium and the extent of its uptake in plants is sometimes related to the activity of VAM fungi, the level of sodium present in the medium however is quite low (1.96 mg/1), therefore, root growth was not adversely affected. Moreover significantly low recovery of root biomass in case of $NaHCO_3$ may be due to increase in level of Na above the critical level.

De-ionization of phytagel with KOH definitely increases the K level in the resultant product. K^+ uptake by any plant is strongly influenced by the form of nitrogen available (whether NO_3 or NH_4^+) as well as by the cations, particularly Na^+. It might also be expected to be influenced by the metabolism and polymerization of phosphate (Harley and Smith 1983). Increased K^+ concentration in the M medium drastically decreases percent colonization of VAM fungus (Becard and Fortin 1988) and high K^+ content was also found to be detrimental to root growth (Harley and Smith 1983). De-ionization of phytagel with NH_4OH also failed to support the root growth. There was no significant difference between NH_4OH and $NaHCO_3$ treatments. The significant reduction may be due to the fact that excess ammonium

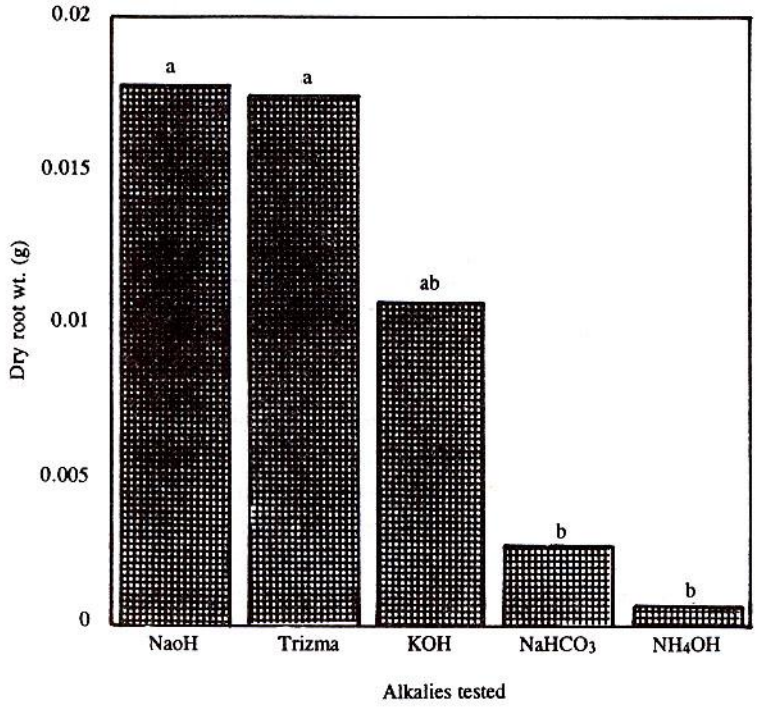

Fig 2. Test of different alkalies for phytagel de-ionization

Those bars followed by the same letter do not differ significantly by Duncan's multiple range test (P<0.01)

ions in M medium result in a rapid drop in the pH of the culture medium and is detrimental to root growth (Becard and Piche 1992).

In summary, concentration of major and minor elements, pH, temperature and the biochemical or physiological demands of the tissues of the test species play a critical role in the root growth. A balance must be reached between the requirements of actively growing roots, which need a complex medium, and those of the extra-radical phase of the VAM fungus, which normally grows in a rhizosphere (i.e. in a relatively nutrient-poor medium).

The present study puts forth a major challenge in the task of identifying appropriate nutritional and physiological demands of root cultures and VAM fungus to develop a strong symbiosis. The impurities identified hi "Phytagel" makes the task of identifying an optimal defined medium much more complex. Therefore, there is a need to look for an alternative while defining the requirement for VAM symbiosis.

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