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In vitro propagation of plant virus using different forms of plant tissue culture and modes of culture operation

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ABSTRACT

Plant virus accumulation was investigated *in vitro* using three different forms of plant tissue culture. Suspended cells, hairy roots and shooty teratomas of *Nicotiana benthamiana* were infected with tobacco mosaic virus (TMV) using the same initial virus:biomass ratio. Viral infection did not affect tissue growth or morphology in any of the three culture systems. Average maximum virus concentrations in hairy roots and shooty teratomas were similar and about an order of magnitude higher than in suspended cells. Hairy roots were considered the preferred host because of their morphological stability in liquid medium and relative ease of culture. The average maximum virus concentration in the hairy roots was $0.82 \pm 0.14\,mg\,g^{-1}$ dry weight; viral coat protein represented a maximum of approximately 6% of total soluble protein in the biomass. Virus accumulation in hairy roots was investigated further using different modes of semi-continuous culture operation aimed at prolonging the root growth phase and providing nutrient supplementation; however, virus concentrations in the roots were not enhanced compared with simple batch culture. The relative infectivity of virus in the biomass declined by 80–90% during all the cultures tested, irrespective of the form of plant tissue used or mode of culture operation. Hairy root cultures inoculated with a transgenic TMV-based vector in batch culture accumulated green fluorescent protein (GFP); however, maximum GFP concentrations in the biomass were relatively low at $39\,\mu g\,g^{-1}$ dry weight, probably due to genetic instability of the vector. This work highlights the advantages of using hairy roots for *in vitro* propagation of TMV compared with shooty teratomas and suspended plant cells, and demonstrates that batch root culture is more effective than semi-continuous operations for accumulation of high virus concentrations in the biomass.

1. Introduction

Plants offer significant advantages in safety and cost over alternative eukaryotic foreign protein expression systems such as mammalian cells and transgenic animals (Daniell et al., 2001; Twyman et al., 2003; Ma et al., 2005). Methods for transient expression of foreign proteins in plants using infection with plant-virus-based vectors have attracted considerable attention (Turpen, 1999; Cañizares et al., 2005; Grill et al., 2005; Lico et al., 2008). Viral infections are initiated by inoculating plants with assembled virus or infectious RNA transcripts containing foreign genes so that foreign proteins are produced as viral replication occurs in the plant tissue. Vectors derived from different plant viruses have been employed to synthesise a variety of medically-related proteins such as vaccines and antibodies. However, research into foreign protein production using plant viral systems has been limited mainly to the use of whole plants.

Plant tissue culture is an alternative technology for foreign protein production (Doran, 2000; Hellwig et al., 2004). Tissue culture systems offer better environmental containment than whole plants grown in the field so that regulatory requirements can be more easily met; production times are also shorter and, for proteins that are secreted from the cells, downstream processing and product purification are simpler and cheaper (Doran, 2006). Most research in this area has focused on the use of undifferentiated plant cultures such as cell suspensions rather than roots and shoots. Yet, hairy roots and shooty teratomas have been demonstrated to be effective host culture systems for recombinant protein synthesis (Wongsamuth and Doran, 1997; Sharp and Doran, 2001a,b; Jin et al., 2005), with potential advantages including greater long-term stability of transgene expression (Sharp and Doran, 2001b). Hairy roots are characterised by fast growth rates compared with whole plants and are more genetically stable than suspended cells (Aird et al., 1988; Flores, 1987). In recent work, hairy roots were found to support plant virus replication, but at levels somewhat lower than

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those often measured in the leaves of whole plants (Shadwick and Doran, 2007a). This raises the question of whether leaf or shoot tissue is more effective for viral infection than roots, which are not a common target for plant virus inoculation and expansion *in vivo*.

This work was aimed at evaluating virus accumulation in suspended plant cells, hairy roots and shooty teratomas for selection of a suitable culture type for subsequent *in vitro* viral infection studies. The relationship between virus replication, nutrient availability and growth of the host plant cells was also investigated using different modes of culture operation including batch and two types of semi-continuous, draw-and-fill culture systems. Tobacco mosaic virus (TMV) was chosen for this work because it is well characterised, has a wide host range, does not require specific insect, nematode or microbial vectors for infection of plant cells, and has been genetically modified for transient expression of several foreign proteins in plants (Shaw, 1999; van den Heuvel et al., 1999; Grill et al., 2005). A transgenic TMV-based vector was used to demonstrate *in vitro* transient expression of a reporter gene, green fluorescent protein (GFP), in hairy root cultures.

2. Materials and methods

2.1. Plant cultures and plants

Hairy roots of *N. benthamiana* were initiated previously using *Agrobacterium rhizogenes* strain A4 (Shadwick and Doran, 2007a). The roots were grown in liquid Gamborg's B5 medium with minimal organics (Sigma–Aldrich, USA) containing 30 g l^{-1} sucrose and with pH adjusted to 5.8 before autoclaving. Hairy roots were cultured in 250-ml shake flasks in the dark at 25 °C on orbital shakers operated at 100 rpm. Shooty teratomas of *N. benthamiana* were initiated from seedlings by infection with *Agrobacterium tumefaciens* strain C58 as described previously (Subroto et al., 1996). Individual shoots formed at each wound site were cultured separately and regarded as a single clone. Liquid Murashige and Skoog (MS) medium (MP Biomedicals, USA) with 30 g l^{-1} sucrose, pH 5.8, was used for shooty teratoma culture. Shooty teratomas were incubated at 25 °C in 250-ml flasks on orbital shakers operated at 80 rpm under continuous fluorescent lighting at an intensity of about 650 lx. *N. benthamiana* callus was initiated from hairy roots using MS medium containing 30 g l^{-1} sucrose, 0.2 mg l^{-1} 2,4-dichlorophenoxyacetic acid (2,4-D), 0.1 mg l^{-1} kinetin and 0.8% agar, pH 5.8. Suspension cultures developed from callus in liquid medium were incubated in the dark at 25 °C on orbital shakers operated at 100 rpm.

Whole plants of *N. tabacum* var. Hicks were used for maintaining stocks of TMV and *N. glutinosa* plants were used for local lesion assays. Seeds were surface sterilised in 4% (v/v) bleach solution containing 0.05% (v/v) Tween 80 for 15 min, germinated on solid Gamborg's B5 medium, and transplanted to sterile Thrive seed raising potting mix (Yates, Australia). *N. tabacum* plants were cultivated in a glasshouse under ambient conditions and *N. glutinosa* plants were grown at 25 °C under continuous fluorescent lighting at an intensity of about 650 lx. Thrive all purpose soluble fertiliser (Yates) was applied weekly at a concentration of 1.8 g l^{-1}.

2.2. Wild-type and transgenic virus

Stocks of purified TMV strain U1 were generated by propagation in *N. tabacum* plants as described previously (Shadwick and Doran, 2007a).

The 30B-based viral vector containing the gene for cycle 3 GFP was supplied in plasmid form by Professor Bill Dawson and Dr Shailaja Rabindran, Department of Plant Pathology, University of Florida, USA. The vector contains the 5′ non-translated region (NTR), the 5′ open reading frame (ORF) encoding two replicase proteins and the ORF for movement protein from TMV strain U1. Heterologous sequences from tobacco mild green mosaic virus encode the coat protein sub-genomic mRNA promoter, coat protein ORF and 3′ NTR. Foreign protein is expressed as free cytosolic protein (Shivprasad et al., 1999).

Transformation of *Escherichia coli* XL1-Blue cells (Stratagene, La Jolla, USA) was performed using the method of Sambrook and Russell (2001). Plasmid extraction was carried out using a QIAprep Miniprep Kit (Qiagen, Venlo, The Netherlands) following the protocol for purifying low-copy plasmids. Plasmid 30B-GFPC3 was linearised by *Kpn I* (Invitrogen, Mount Waverley, Australia) and plasmid purification was carried out using a QIAquick PCR purification kit (Qiagen). Capped infectious viral RNA was generated by *in vitro* transcription using a mMESSAGE mMACHINE® high yield capped RNA transcription kit (Ambion, Austin, USA) according to the manufacturer's protocol with an additional 1 μl GTP (30 mM) included in the reaction mixture and an incubation time of 2 h. RNA transcript was purified using a MEGAclear™ purification kit (Ambion).

To produce assembled transgenic TMV-GFPC3 virus, two healthy opposite leaves located on the lower stem of *N. benthamiana* plants were selected for inoculation and sprinkled with a small amount of sterile 500-mesh carborundum powder (Naxos Products, Auburn, Australia). The leaves were inoculated with RNA transcript, rubbed once gently with a gloved forefinger and rinsed with sterile water. After 21 days of glasshouse cultivation, most upper, non-inoculated leaves showing symptoms of viral infection were harvested.

TMV and TMV-GFPC3 were purified from plant material using methods modified from Gooding and Hebert (1967) as described for purification of tomato mosaic virus in C.M.I./A.A.B. Description Plant Viruses (Association of Applied Biologists: http://www.dpvweb.net/dpv/showdpv.php?dpvno=156). Purified virus preparations were filter-sterilised (0.2 μm, Sartorius Minisart, Germany).

2.3. Virus propagation using different forms of plant tissue culture

Hairy root cultures were initiated by inoculating 0.2 g fresh weight of roots from 21-day-old cultures into 250-ml shake flasks containing 50 ml of medium. The roots were infected with 75 μg of purified TMV diluted in sterile 0.01 M phosphate buffer to give an initial concentration in the medium of 1.5 μg ml^{-1}. Virus was added to the roots at the same time as the roots were inoculated into fresh medium. The same volume of 0.01 M phosphate buffer without TMV was added to negative control cultures.

Shooty teratoma cultures were initiated by inoculating 1.2 g fresh weight of shoots from 28-day-old cultures into 250-ml shake flasks containing 50 ml of medium. A greater amount of shoot biomass is needed to initiate shooty teratoma cultures than is required for subculture of hairy roots. To maintain the same initial virus:biomass ratio as in the hairy root cultures, 450 μg of purified TMV was added to give an initial TMV concentration of 9.0 μg ml^{-1}.

Cell suspension cultures were initiated by transferring 10 ml of inoculum from 12-day-old cultures into 40 ml of medium in 250-ml shake flasks. To provide the same initial virus:biomass ratio as in the hairy root and shooty teratoma cultures, 300 μg of purified TMV was added to give an initial TMV concentration of 6.0 μg ml^{-1}.

Triplicate or quadruplicate cultures were harvested periodically for measurement of biomass, virus concentrations in the biomass and medium, total soluble protein concentration in the biomass, and virus infectivity.

2.4. Virus accumulation during batch and semi-continuous cultures of hairy roots

To investigate whether prolonging root growth could increase virus concentrations in the biomass, hairy roots were cultured using semi-continuous, draw-and-fill culture systems. Flasks were each