Plant Molecular Biology Reporter
Volume 9(1) 1991

pages 13-20

Protocol

Monitoring Hairy-Root Growth by Image Analysis

Graeme D. Coles, D. J. Abernethy, Mary C. Christey, A. J. Conner, and B. K. Sinclair

DSIR Crop Research, Private Bag, Christchurch, New Zealand

Key Words: Image processing, hairy roots, *Agrobacterium rhizogenes*, growth analysis.

Abstract: Hairy roots, incited by *Agrobacterium rhizogenes*, form a useful system for analysing the expression and phenotypic effects of foreign genes in plant root tissue. Image analysis offers a non-invasive method of describing their growth in culture. Images of pea (coarse) and *Brassica* (fine) hairy roots were captured, processed and analysed without difficulty using a commercially available image analysis system. The value of this method in monitoring intermediate changes in growth pattern was illustrated by following the changes in five putatively chlorsulfuron-resistant *Brassica* hairy-root lines cultured with and without a selective level of chlorsulfuron. Areas of hairy-root research where this technique will be particularly useful are discussed.

Agrobacterium rhizogenes incites the proliferation of hairy roots on plants via a natural genetic engineering event. This soil bacterium infects wound sites on plants and transfers specific regions of its Ri (root-inducing) plasmid, the T-DNA, into the genomic DNA of plant cells (Birot et al., 1987). The resulting transformed cells develop into hairy roots due to the expression of T_L *rol* loci, which are thought to sensitise plant cells to endogenous auxins (Gelvin, 1990). Expression of T_R auxin biosynthetic genes may also play a minor role in hairy-root development.

Abbreviations: LS medium, Linsmaier and Skoog medium; T-DNA, transferred DNA; T_L, left transferred DNA; T_R, right transferred DNA; d.f., degrees of freedom; T_i, tumor-inducing plasmid; R_i, hairy-root-inducing plasmid.

Hairy roots can be readily induced *in vitro* on a wide range of dicotyledonous plants (Tepfer et al., 1988). They can be repeatedly excised and transferred to hormone-free culture medium, on which they proliferate into a highly branched, plagiotropic root system that rapidly spreads over the surface of solidified culture media. By using *Agrobacterium rhizogenes* strains bearing binary vectors, transformed hairy roots can be selected which express foreign genes (e.g., Grant et al., 1990; Christey and Sinclair, 1990). This offers a convenient approach for rapidly assessing the expression and value of transferred genes in the root systems of a range of species.

Hairy roots can also form substrates for studying interactions with other organisms such as nematodes (Verdejo et al., 1988), mycorrhizal fungi (Mugnier and Mosse, 1987), root nodule bacteria (Petit et al., 1987; Hansen et al., 1989) and various pests and pathogens (Mugnier 1987). They therefore offer a valuable system for studying the effect of expressing foreign genes on interactions between plant roots and other organisms.

To use hairy-root systems effectively in plant science research, a convenient means for monitoring their growth is needed. Simple estimates of hairy-root mass are difficult due to problems in separating solidified culture medium from the highly ramified hairy-root systems. Furthermore, it is clearly possible that only measuring tissue mass at the start and finish of a growth experiment could conceal very interesting behaviour during the course of that experiment. A non-invasive method for estimating culture size would allow the examination of short-term effects while the experiment is in progress. Image processing and analysis appears to offer a method to estimate culture size non-invasively.

Image processing is the capture of an analogue video image, its subsequent conversion to a digital representation, and the segmentation of the image into its features of interest and the background. Image analysis is the process of extracting information about the features of this segmented image (Rosenfeld, 1988; Jarvis, 1988). The aim of the experiments reported here was to demonstrate that a standard image processing and analysis system could be used to measure the growth of hairy-root cultures non-destructively and at intervals during the course of an experiment.

Materials and Methods

The Image Processor

Image processing and analysis were carried out using the Chromatic colour image analysis system (Leading Edge Pty, 28 University Way,

Bellevue Heights, South Australia 5050). The image capture environment consisted of a diffuse source of backlighting, a JVC TK870E colour video camera head with a Cosmicar ES-4 25-mm autoiris lens and the PIB+ digitising frame store from Atronics International (1830 McCandless Drive, Milpitas, California 95035, USA). The host computer had an 80386 processor running at 20MHz, VGA standard Graphics card and its data bus provided two free adjacent eight-bit slots into which to install the frame store. A Centronics standard printer port was required to carry the software multiuse prevention device used by the Chromatic system. All operations were carried out in a darkened room, and the light source was masked to prevent stray light entering the lens.

Hairy-root lines

This study involved hairy-root lines of *Pisum sativum* cv Whero (line WHR73), *Brassica napus* cv Giant (lines GHR, (GHR7) and *Brassica campestris* cv Red Globe (lines RGHR, RGHR3 and RGHR4), originating from previous studies (Grant et al., 1990; Christey and Sinclair, 1990). All hairy roots were selected following cocultivation of seedling tissue with *Agrobacterium* strain A4T (C58C1, pRiA4) (Moore et al., 1979), harbouring the binary vector pKIWI110 (Janssen and Gardner, 1990). This vector has three genes that can be transferred to and expressed in plant cells. These confer kanamycin resistance, glucuronidase activity, and chlorsulfuron resistance. The hairy roots were maintained on hormone-free LS medium (Linsmaier and Skoog, 1965), supplemented with cefotaxime (200 mg/L) and kanamycin (20 mg/L for peas and 50 mg/L for *Brassica*), and solidification with 25 g/L of gelrite (for *Brassica*) and 8 g/L Davis agar (for peas).

Measurement of hairy-root cultures

Images of hairy roots were captured through the bottom of the container and through the medium to eliminate problems caused by condensation on the container lid. The threshold for segmentation is used to produce a binary overlay on the digitised image, where for each pixel meeting the threshold criterion, the overlay is *on*. The threshold was achieved by choosing the lightest point within the image that lay on the culture. Areas of the overlay switched on over features not part of the culture were removed by a built-in image editor, while 'holes' in the overlay over areas of interest were filled using the binary editor. The objective was an overlay congruent with the hairy-root culture beneath.

Measurements were then made on the overlay and on the pixels

underlying it, using algorithms provided with the software. Area and integrated optical density were both recorded, but since these values were highly correlated ($r^2 = 0.89$, d.f. = 28), only area measurements were used.

Data are reported as relative areas, i.e.

$$\text{Relative area}_{ti} = \text{area}_{ti}/\text{area}_{to}$$

where area_{ti} is the area of the overlay at the time of interest, and area_{to} is the area of the overlay at the start of the experiment.

Experimentation

Fragments of hairy roots of pea and *Brassica* were inoculated on to the centre of plastic petri dishes (90-mm diameter and 15-mm depth) containing 25 mL of LS medium plus cefotaxime (200 mg/L). Five replicates of each line were established. Care was taken to ensure that the culture fragment was firmly embedded in the medium, and that no fragments of previous medium were transferred. A first measurement was made immediately after transfer and subsequent measurements were made at two-day intervals.

In order to demonstrate the utility of interim measurements in obtaining maximum value from experiments on hairy roots, the growth of five independently selected *Brassica* hairy-root lines was monitored in the presence and absence of chlorsulfuron. Hairy roots were transferred to the centre of plastic pottles (90-mm diameter and 50-mm depth) containing 50 mL of LS medium plus cefotaxime (200 mg/L) and two levels of chlorsulfuron (0 and 100 µg/L). Measurements were made twice weekly.

Results and Discussion

Hairy-root lines of pea and *Brassica* used in this study were specifically chosen for their contrasting morphology. They represent the extremes of growth form usually found in hairy roots. Whereas the *Brassica* hairy roots were very fine and highly branched (Fig. 1 left), the pea hairy roots were very coarse, with less branching, as a result of greater apical dominance (Fig. 1 right). For both of these growth forms, the overlay produced by image processing is congruent with the hairy-root culture. (Compare Figs. 1 and 2 left and 1 and 2 right).

The relative growth rates of pea and *Brassica* hairy roots estimated by image analysis is presented in Figure 3. It is clear that this technique has been able to monitor hairy-root growth throughout the culture

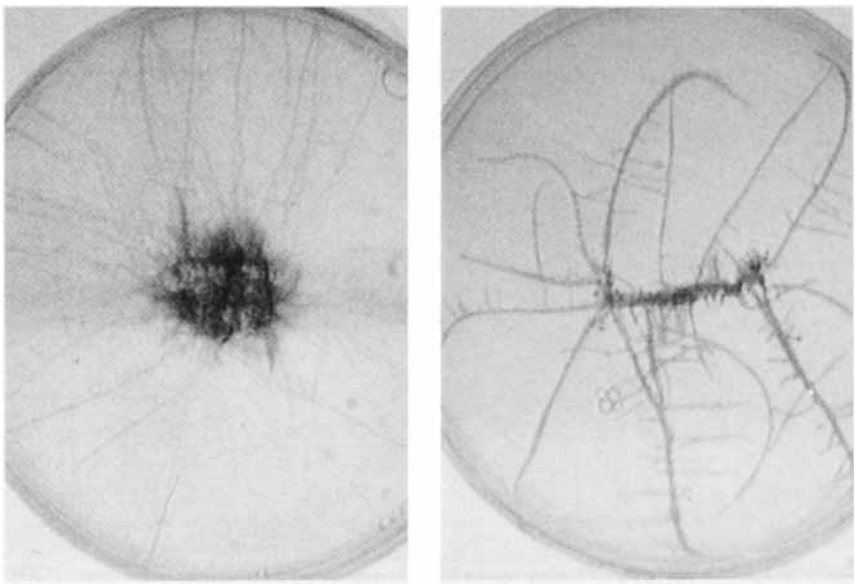

Fig. 1. Hairy roots in culture, as presented to the Image Analysis system. (left) *Brassica*, (right) pea.

period, and therefore allow estimation of growth rate. The levelling off of the pea growth rate is due to the hairy roots completely filling the petri dish after 10 days.

The growth response of five independently selected *Brassica* hairy-root lines to media with and without chlorsulfuron is presented in Figures 4. In the absence of chlorsulfuron the hairy-root lines showed different growth patterns in culture (Fig. 4 top). These hairy-root lines also showed different growth responses in the presence of 100 µg/L of chlorsulfuron (Fig. 4 bottom), enabling us to distinguish resistant and sensitive lines. The time at which rapid growth commenced varied between the hairy-root lines, which would not have been detected if measurements were only made at the beginning and end of the experiment.

Conclusion

It is possible to follow growth of hairy-root cultures in a non-invasive manner using image processing and analysis. This method is very rapid

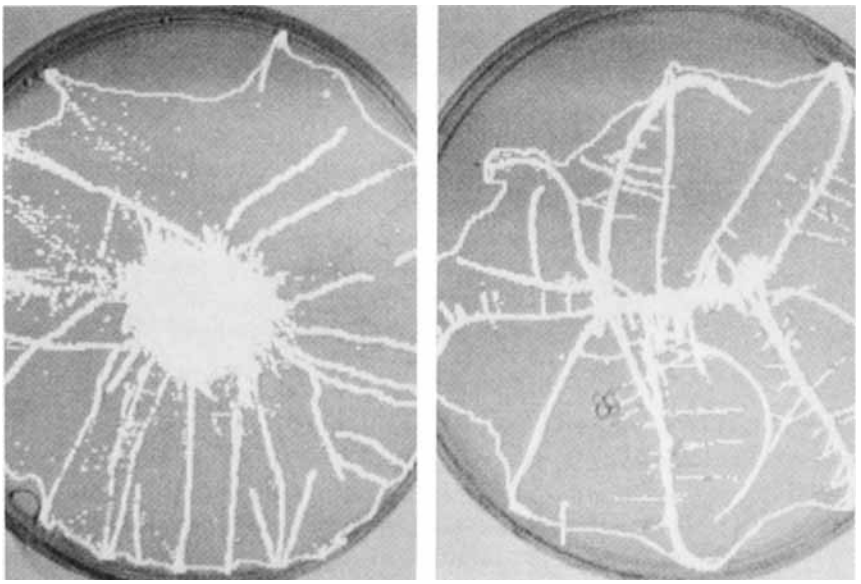

Fig. 2. Edited overlays of hairy roots in culture, ready for measurement. (left) *Brassica*, (right) pea.

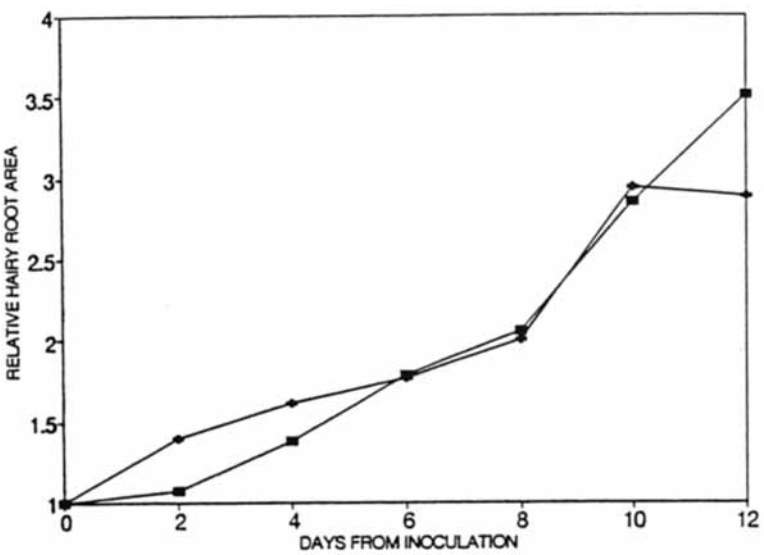

Fig 3. Relative size of pea (crosses) and *Brassica* (solid boxes) hairy roots with time. The pea cultures were inhibited from further growth by reaching the sides of the container sooner than *Brassica*, due to their higher apical dominance.

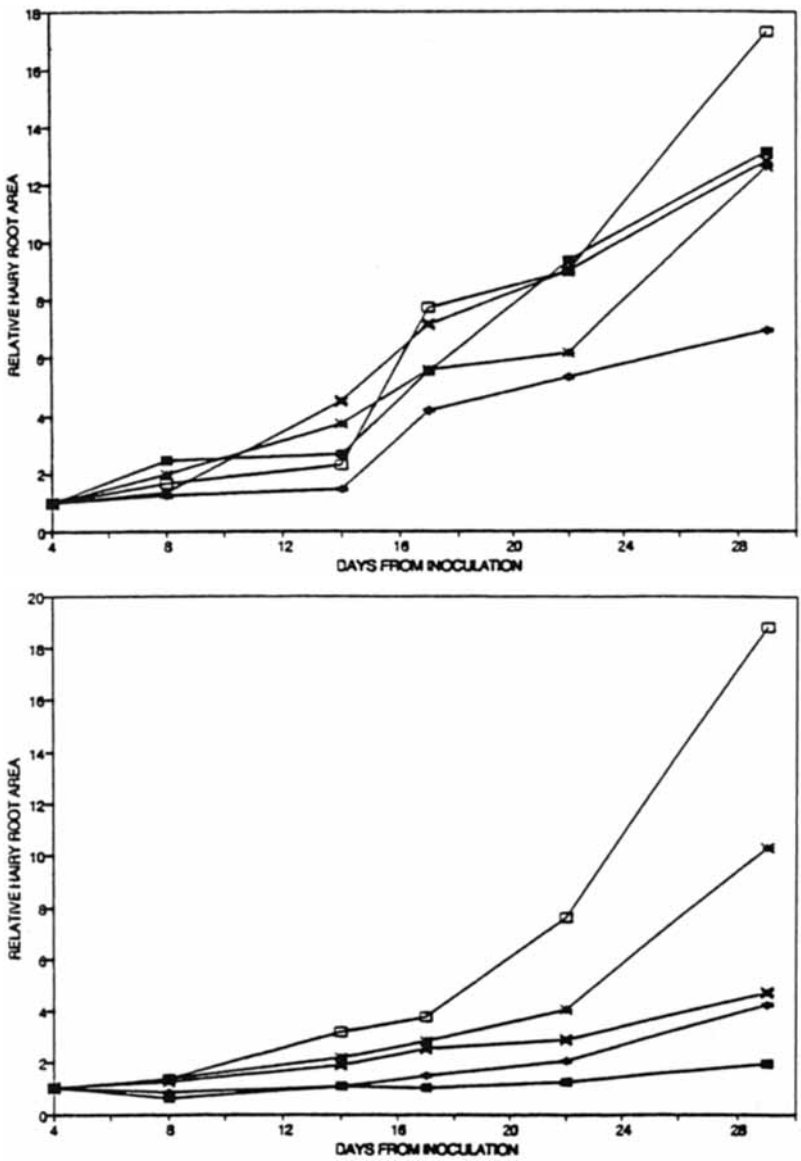

Fig. 4. Comparison of behaviour in culture for five independently selected *Brassica* hairy-root lines. Closed square: GHR; cross: GHR7; asterisk: RGHR; open square: RGHR3; St Andrew's cross: RGHR4. (top) No chlorsulfuron, (bottom) 100 µg/L chlorsulfuron.

and repeatable and, since the capital cost can be spread over many thousands of samples, the cost of individual measurements is minimal. Image analysis does not require disturbance of the culture, so estimates of growth are not confounded by artifacts of the measurement method. Training costs are low, and the equipment and software are robust. This nondestructive method allows measurements to be repeated as often as necessary during the course of an experiment.

This approach for measuring hairy-root growth will be valuable for assessing the phenotypic effects of expressing foreign genes in plant root systems, especially genes affecting ion uptake and utilisation, resistance to heavy metals, and interactions with beneficial and pathogenic microbes and invertebrates.

References

Birot, A. M, D. Bouchez, F. Casse-Delbart, M. Durand-Tardif, L. Jouanin, V. Pautot, C. Robaglia, D. Tepfer, M. Tepfer, J. Tourneur and F. Vilaine. 1987. Studies and uses of the Ri plasmid of *Agrobacterium rhizogenes*. Plant Physiol. Biochem. 25:323-335.

Christey, M. C. and B. K. Sinclair. 1990. Selection of transformed hairy-root lines in *Brassica oleracea*, *B. napus* and *B. campestris*. Proceedings of the 6th Crucifer Genetics Workshop, Cornell University, Ithaca. *in press*.

Gelvin, S.B. 1990. Crown gall disease and hairy-root disease. A sledgehammer and a tackhammer. Plant Physiol. 92:281-285.

Grant, J. E., G. M. Timmerman and A. J. Conner. 1990. Genetic engineering for grain legume improvement. New Zealand Agronomy Society Special Publication No.7, *in press*.

Hansen, J., J. E. Jorgensen, J. Stougård, and K. A. Marcker. 1989. Hairy roots–a short cut to transgenic root nodules. Plant Cell Reports 8:12-15.

Janssen, B. J., and R. C. Gardner. 1990. Localised transient expression of GUS in leaf discs following cocultivation with *Agrobacterium*. Plant Mol. Biol. 14:61-72.

Jarvis, L. R. 1988. Microcomputer video image analysis. J. Micros. 150:83-97.

Linsmaier, E. M. and F. Skoog. 1965. Organic growth factor requirements of tobacco tissue culture. Physiol. Plant. 18:100-127.

Moore, L., G. Warren and G. Strobel. 1979. Involvement of a plasmid in the hairy-root disease of plants caused by *Agrobacterium rhizogenes*. Plasmid 2:61-72.

Mugnier, J. 1987. Infection by *Polymyxa betae* and *Plasmodiophora brassicae* of roots containing root inducing transferred DNA of *Agrobacterium rhizogenes*. Phytopath. 77:539-542.

Mugnier, J. and B. Mosse. 1987. Vesicular–arbuscular mycorrhizal infection in transformed root–inducing T–DNA roots grown axenically. Phytopath. 77:1045-1050.

Petit, A., J. Stougård, A. Kühle, K. A. Marcker and J. Tempe. 1987. Transformation and regeneration of the legume *Lotus corniculatus*: A system for molecular studies of symbiotic nitrogen fixation. Mol. Gen. Genet. 207:245-250.

Rosenfeld, A. 1988. Image analysis and computer vision. Computer Vision, Graphics and Image Processing. 46:196-264.

Tepfer, D., L. Metzger, and R. Prost. 1989. Use of roots transformed by *Agrobacterium rhizogenes* in rhizosphere research: applications in studies of cadmium assimilation from sewage sludges. Plant Mol. Biol. 13: 295-302.

Verdejo, S., B.A. Jaffee and R. Mankau. 1988. Reproduction of *Meloidogyne javanica* on plant roots genetically transformed by *Agrobacterium rhizogenes*. J. Nematol. 20:599-604.