The Regeneration of Virus-Free Plants from Cucumber Mosaic Virus- and Potato Virus Y-Infected Tobacco Explants Cultured in the Presence of Virazole

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Adventitious shoots regenerated from tobacco explants taken from donor-plants infected with cucumber mosaic virus and potato virus Y, were virus infected. Similar explants cultured in the presence of Virazole were virus-free.

The strategy for mass cloning of plants by micropropagation depends on disease-indexing the donor plant, and eliminating any diseases found, before setting up the explant or meristem cultures. Details of tissue culture media have been published for most commercially important species [1]. Fungal, bacterial and mycoplasmal pathogens can be successfully eliminated by the addition of the appropriate antibiotics to the culture medium, but the potential of micropropagation will be realized only when virus elimination can be easily achieved.

Presently, virus exclusion from the meristem and/or differential thermal stability of virus and host tissue, are used to produce virus-free "elite" stock plants for subsequent cloning [2]. However, these procedures are not universally efficaceous, some viruses being particularly invasive or having relatively high thermal stability [3, 4]. Furthermore, low virus titre or slow systemic movement may necessitate repeated virus testing of the progeny plant before one can be reasonably confident of virus elimination and during this time there is risk of reinfection.

Many of the potential antiviral chemicals developed by animal virologists have also been screened in plants but results so far have not been encouraging [5]. Much of this work is speculative in plants, since many of the chemicals are uncharacterized inhibitors or were designed putatively, as inhibitors of functions of specific animal viruses eg replicases. The latter may lack wide spectrum activity required for the commercial control of virus diseases of plants occurring in the field, which often result from mixed viral infection. In addition, as site specific inhibitors, such chemicals are potentially too expensive for field application to plants and this is compounded, for as reversible inhibitors, repeated applications would be required as the crop develops, and for perennial crops.

Micropropagation presents a new system for the evaluation of antiviral chemicals in plants because of the nature of the process. Proliferation of organized tissue occurs in meristem cloning; adventitious shoot or vegetative embryo formation may be induced directly from explants or from callus derived from them. Furthermore, the cost benefits to be derived from micropropagation can support the use of potentially expensive chemicals since milligramme quantities are involved (contrast with the amounts required for field application).

In this communication we report the results of our studies on virazole (1-β-D-ribofuranosyl-1,2,4-triazole-3-carboxamide (ICN Pharmaceuticals Inc., Cleveland, Ohio, USA) a novel antiviral chemical which is reported to inhibit host functions in the late stages of virus maturation [7].

Seedlings of *Nicotiana tabacum* cv. xanthi-nc were grown as described [6] and mechanically inoculated at the 3–4 leaf stage with either potato virus Y (PVY) or cucumber mosaic virus (CMV) (wild stains). Petioles were excised from the systemically infected plants when they reached the 12–14 leaf stage (approx. 35 cm in height) and surface sterilized in 80% aq. ethanol for 1 min followed by immersion in hypochlorite solution for 15 min (10% aq. Domestos; Lever Bros., Liverpool, U. K.). The petioles were rinsed in sterile distilled water and placed on the tissue culture medium which consisted of the minerals, trace elements and vitamins of Murashige and Skoog [8] plus 3% w/v sucrose, 9.4 µM zeatin and 0.8% w/v agar, pH 5.8. Virazole was added after autoclaving, by sterile filtration. Autoclaving virazole reduced its activity. Explants were cultured in a growthroom provided with a 16 h photoperiod, the light intensity was 5 Wm^{-2} and the temperature 22 °C.

Twelve replicate cultures were set up for each virazole concentration. Shoots were taken at random from these cultures when they reached approx. 1 cm

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in length. The time of sampling varied with the concentration of virazole used in the medium, from 6 weeks at 20.5 μM virazole, to approx. 22 weeks when cultured on 205 μM virazole. Concentrations in excess of 205 μM were toxic. Virazole at 205 μM decreased the rate of adventitious shoot growth but increased shoot numbers.

Shoots were excised from the explants and placed directly in unsterilized potting compost on a propagating bench in insect-proof boxes. After rooting (approx. 2–3 weeks later) the plantlets were potted on and grown in insect-proof cages. When the plants reached the four leaf stage, they were screened for virus. CMV was tested on *Chenopodium amaranticolor* by local lesion assay and PVY by leaf dip electron microscopy [9]. Plants which gave a negative reaction were grown on to the 14 leaf stage and retested.

Explants from both PVY and CMV infected donor plants cultures in the absence of virazole, gave rise to adventitious shoots, all of which were infected. This result has been obtained repeatedly. PVY-infected explants, cultured in the presence of 20.5, 41 and 205 μM virazole, produced adventitious shoots which were 99%, 87.5% and 0% infected respectively, while CMV-infected explants cultures on the same concentrations of virazole produced adventitious shoots which were 79%. 74% and 0% infected respectively. When infected explants were transferred after bud formation on medium containing 205 μM virazole, to virazole-free medium, prior to excising the shoots, the percentage of infected shoots increased from 0% to 44% and 33% for PVY and CMV respectively. In interpreting the data (Table I), it should be noted that virus transmission from infected to non-infected adventitious shoots is possible in the cultures before and during excision and rooting.

Previous studies have suggested that virazole may inhibit a host function affecting virus maturation [7]. In the present work, and in work on the regeneration of plants from protoplasts isolated from potato virus X infected tobacco plants [10], it has been shown that virazole in the differentiation medium may result in the production of virus-free adventitious shoots. The effect of virazole has been shown here to be reversible and the data suggest an effect on virus movement into the adventitious buds and/or vegetative embryos from which the adventitious shoots are derived. If variazole acts as proposed here, it may have broad spectrum activity and commercial application in the micropropagation of virus-free stock plants. This latter aspect is being further investigated.

Table I. The percentage of infected adventitious shoots produced in explant culture in the presence of different concentrations of virazole. The explants were taken from tobacco plants systemically infected with either potato virus Y (PVY) or cucumber mosaic virus (CMV) and cultured on Murashige and Skoog medium [8] containing 9.4 μM zeatin [6] (the control), or the appropriate virazole concentration. Following adventitious shoot formation, the individual shoots were excised, rooted in compost and grown to the 4 leaf stage (all operations carried out in insect-proof cages) when they were virus screened. CMV was tested by local lesion assay and PVY by electron microscopy. Plants which were determined to be free of virus were grown on to the 12–14 leaf stage and rescreened. Data are for the mean of replicate experiments. The controls were in all cases 100% infected.

Virus	Virazole conc. [μM]	No. of plants screened	% Virus infected
PVY	20.5	52	99
	41.0	48	87.5
	205.0	19	0
	205.0	43 [a]	44 [a]
CMV	20.5	69	79
	41.0	54	74
	205.0	16	0
	205.0	77 [a]	33 [a]

[a] Partial replicate experiment in which the explants were cultured on virazole containing medium until bud formation, then transferred to virazole medium before subsequent excision of the shoots.

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