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Regeneration of *Rubus in vitro* using forchlorfenuron (CPPU)[1]

Regeneración *in vitro* de *Rubus* usando forclorofenuron (CPPU)

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Abstract

The ability of N-(2-chloro-4-pyridyl)-N-phenylurea (CPPU), a synthetic compound with cytokinin-like activity, to induce regeneration of whole plants in *Rubus* was assessed. Regeneration was obtained from internodal segments of three tissue culture recalcitrant red raspberry cultivars Glen Prosen (32%), Glen Moy (38%) and Glen Magna (72%) using media containing low concentrations of CPPU. The ability of CPPU to induce regeneration from the cultivars Tayberry (38%) and Loch Ness (46%) was examined and the results compared with previous work. The regeneration protocol here tested provides the first step for future genetic transformation involving these three *Rubus* varieties.
Key words: CPPU, *Rubus*, organogenesis, regeneration.

Resumen

N-(2-chloro-4-pyridyl)-N-phenylurea (CPPU), un compuesto sintético con actividad similar a la citocinina fue utilizado para evaluar la habilidad de inducir regeneración completa *in vitro* de *Rubus*. La regeneración fue obtenida a partir de segmentos internodales de tres variedades recalcitrantes de moras, cultivares Glen Prosen (32%), Glen Moy (38%), Glen Magna (72%) usando medios de cultivo con bajas concentraciones de CPPU. La habilidad de CPPU para inducir regeneración *in vitro* de los cultivares Tayberry (38%) y Loch Ness (46%) fue también estudiada y los resultados comparados con trabajos previos. El protocolo de regeneración aqui presentado ofrece el primer paso para futuras transformaciones geneticas de estas tres variedades de *Rubus*.
Palabras Claves: CPPU, *Rubus*, organogenesis, regeneración

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Introduction

The regeneration of whole plants from cultured tissues is a prerequisite for the application of a number of techniques. Modern genetic approaches to crop improvement such as *Agrobacterium*-mediated transfer, protoplast fusion, electroporation, microprojectile bombardment, or microinjection depend on the availability of an efficient regeneration step. These transformation systems are based on the capacity of a single or a few cells, into which foreign DNA has been introduced, to regenerate to a whole plant (11).

Woody plants have proven to be difficult to regenerate *in vitro* becoming a limiting factor in any genetic transformation process, which would provide great benefit to many woody species.

The development of efficient and widely applicable regeneration systems has been possible but difficult and inefficient in *Rubus* spp. thus limiting the application of novel approaches to plant improvement in this genus.

The genus *Rubus* is one of the most diverse in the plant kingdom. The *Idaeobatus* subgenus containing the European red raspberry (*Rubus idaeus* subsp. *vulgatus* Arrhen.), the North American red raspberry (*R. idaeus* subsp. *strigosus* Michx) and the black raspberry (*R. occidentalis* L.), all diploids, and the subgenus *Eubatus* containing the blackberries, with ploidy ranging from diploid (2n=14) to dodecaploid (2n=84) (8) are the most important of some 500 species in the genus *Rubus*.

Improvements in *Rubus* cultivars have been achieved through traditional plant breeding, and although this has been very successful, there are a number of drawbacks associated with the process. These include the timescale involved in obtaining desirable characteristics in a particular cultivar, the limited ability to incorporate a specific character through conventional breeding, the co-transfer of undesirable characters through linkage or pleiotrophic effects and differences in ploidy preventing the production of fertile hybrids. Plant biotechnology, comprising of plant tissue culture and plant molecular biology, has the potential to overcome some of the limitations of plant breeding and thus provide a means of introducing specific improvements and producing new combinations of genetic material in soft fruit.

Adventious shoot regeneration was reported from excised *Rubus* cotyledons (2) which is very time-consuming, and also from leaf discs and internodal stem segments from micropropagated cultures of two red raspberry genotypes (10). However the regeneration efficiency of the raspberry genotypes is low and its capacity to regenerate differ considerably and depends on the explant type used and genotype.

A number of media have been reported for regeneration of *Rubus* and generally with blackberry (*R. fruticosus* L. agg) in their genetic make-up (2, 3, 4, 5, 6, 9, 10, 17), though the efficiency is generally poor

or they are effective on a small number of genotypes. New growth factors, therefore, need to be examined with *Rubus* species, and in particular with *R. idaeus*, the red raspberry, in order to correct these problems.

N-(2-chloro-4-pyridyl)-N-phenylurea (CPPU) is a synthetic compound that has been demonstrated to have cytokinin-like activity (1,18) similar to Thidiazuron (TDZ), one of the most active substances used in *Rubus* tissue culture. The strong cytokinin-like activity of CPPU has been exploited to delay fruit maturity and for increasing fruit size and yield in several fruit crops, including grapes and apples (7, 16). In plant tissue culture its use has been limited despite its potential in regulation of cell division and growth (15).

Ohyama (14) found stimulation of shoot formation on mulberry (*Morus* sp.) hypocotyls cultured *in vitro* with CPPU at a greater efficiency than when BAP was used. CPPU has also been used to facilitate efficient micropropagation of peanut (*Arachis hypogaea* L.), a normally recalcitrant species (13). The use of CPPU in *Rubus* has not been reported.

This paper presents an evaluation of the effectiveness of CPPU on the *in vitro* regeneration of three different red raspberry cultivars for which no regeneration systems are available. The effect of CPPU on a blackberry and hybridberry were also examined for comparison with previous regeneration efficiencies in the presence of a range of growth factors.

Materials and methods

Red raspberry (*R. idaeus*) cultivars Glen Moy, Glen Prosen and Glen Magna, the hybridberry cultivar Tayberry (*R. loganobaccus*) and the blackberry cultivar Loch Ness (*R. fructicosus* L. agg) were maintained in tissue culture on MS medium (12) solidified with agar and containing 20 g/L sucrose and no plant growth regulators. The cultures were grown in growth rooms maintained at 20°C under warm white fluorescent tubes at 70 mmol $m^{-2}s^{-1}$ for a 16-hour day to provide explant material.

Explant material: All explant material was obtained from axenic, vigorous young microplants, on the sixth to eighth week after subculture. Internodal segments of approximately 0.1 cm in length were prepared from all the microplants and the outer tissue removed by peeling under a dissecting microscope in order to expose the cortical tissue directly to the regeneration medium (3,4). Leaf discs were excised using a cork borer to include the mid-vein from the red raspberry cultivars.

A number of replicates containing 50 internodal segments and 20 leaf discs were placed on to the MS regeneration medium containing CPPU (obtained from Sigma) at concentrations of 0.05, 0.1, 0.2, 0.5, 1.0 mg L^{-1} and maintained under the conditions described above.

The results are based on the regeneration efficiency of the plant geno-

type in the specific media concentration. The regeneration efficiency was defined as the percentage of the number of regenerated explants in relation with their initial number in the protocol. Data were analysed using analysis of variance (Minitab, trademark of minitab,Inc.), an easy-to use general purpose statistical computing system.

Results

No regeneration was obtained from leaf discs of the red raspberries at any of the CPPU concentrations. In contrast, shoot production was achieved from internodal segments of all genotypes to various extents, depending on the concentration of CPPU (table 1). The best regeneration efficiency was obtained from 'Glen Magna' where all concentrations of CPPU induced upwards of 40% regeneration (figure 1). In this cultivar 0.5 mg L^{-1} produced the best response with 72% regeneration. Although 0.5 mg L^{-1} CPPU gave the greatest response, 1.0 mg L^{-1} produced plantlets more quickly. With 'Glen Prosen' and 'Glen Moy', only 1.0 mg L^{-1} CPPU was significantly less effective at inducing regeneration than the other concentrations. Here, 0.2 mg L^{-1} for 'Glen Prosen' and 0.1 mg L^{-1} for 'Glen Moy' were optimum.

With 'Tayberry', 0.2 mg L^{-1} CPPU was the optimum concentration (38%), while for 'Loch Ness', 0.5 mg L^{-1} was optimal (46%). These results from 'Tayberry' and 'Loch Ness' however, are lower than previously achieved on other growth factors (3,5,10). Genotypes differed significantly ($P < 0.025$-0.001) in

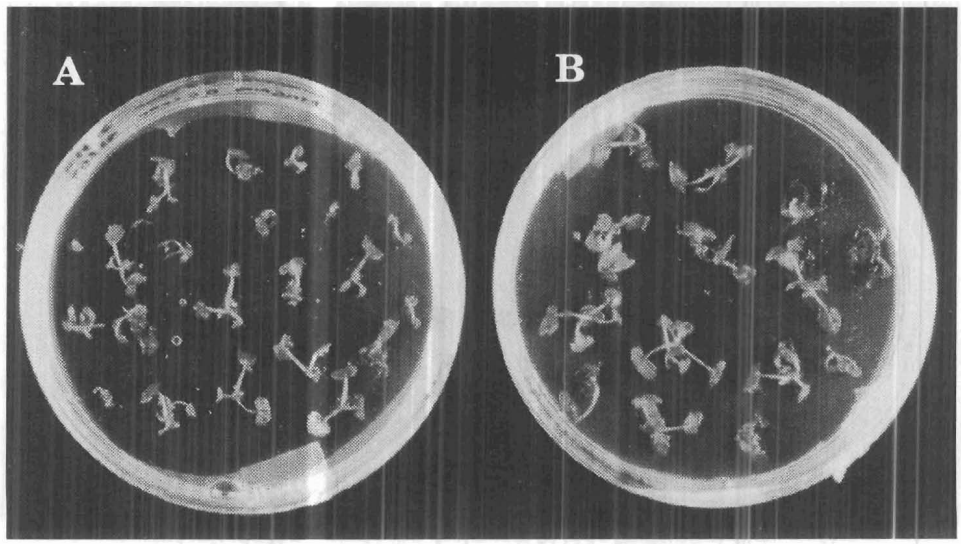

Figure 1. *In vitro* regeneration of *Rubus idaeus* cv. Glen Magna using 1.0 mg L^{-1} of CPPU (A) and 0.5 mg L^{-1} CPPU(B).

Table 1. Regeneration efficiency from internodal segments of five *Rubus* varieties using different CPPU concentrations.

CPPU mg L^{-1}	cv. Glen Prosen %	cv. Glen Moy %	cv. Glen Magna %	cv. Tayberry %	cv. Loch Ness
0.05	26	20	40	4	18
0.1	26	38	42	20	24
0.2	32	28	44	38	28
0.5	28	26	72	12	46
1.0	10	13	60	8	30

their ability to regenerate on four of the five concentrations of CPPU. No significant difference was found on 0.2 mg L^{-1} CPPU. The hormone concentration had a significant effect on the ability of all genotypes to regenerate (Glen Prosen $P < 0.025$, Glen Moy $P < 0.01$, Glen Magna $P < 0.001$, Tayberry $P < 0.001$ and Loch Ness $P < 0.005$).

Discussion

Regeneration from red raspberry has proven difficult. Other *Rubus* spp. have been more amenable to regeneration, especially those with blackberry in their genetic make-up. Some growth factors have induced a small amount of regeneration on the red raspberries, however, there was a need to examine alternative growth substances as the more commonly used ones have been generally unsuccessful and inefficient.

This is the first report of successful and complete regeneration from red raspberry using CPPU. The percentage of regeneration is also higher than that obtained from other growth factors. It also highlights the genotype-specific nature of whole plant regeneration from soft fruit (3). The use of CPPU will be extended to a wide range of red raspberries to investigate how effective it will prove across a wider range of genotypes.

According to the genetic relatedness of the varieties here tested using RAPD markers they can be divided into three groupings. The *Eubatis* or blackberries (Loch Ness), the Glen raspberries and the grouping of the hybrid berry (Tayberry) (5). The results here obtained are according with this grouping and the use of CPPU for *in vitro* regeneration is more effective for the three recalcitrant Glen raspberries than for cv. Loch Ness and cv. Tayberry which have better response with other growth regulators.

The regenerated explants obtained using these concentrations of CPPU do not go through callus stage which is an advantage for the transformation technology in order to avoid somaclonal variation. The development of this regeneration system will allow progress to be made in the application of transformation technology to the above varieties.

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