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LEAF DISC REGENERATION OF PASSION FRUIT

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ABSTRACT

A leaf disc regeneration system was developed for passion fruit, *Passiflora edulis* Sims. Leaf discs were cultured on a modified MS medium containing 8.90 μM BAP or on 8.90 μM BAP and 2.32 μM KIN. Shoots appeared within four weeks. These could be rooted when transferred to the same medium containing 0.54, 2.69 and 5.37 μM NAA. Leaf discs cultured on medium supplemented with 11.42 μM IAA, 10.74 μM NAA and 1.86 μM KIN formed callus only. Those cultured on medium supplemented with 10.74 μM NAA and 2.32 μM KIN formed callus and roots.

***Key Words*: Leaf disc regeneration, passion fruit, *Passiflora edulis*, tissue culture.**

RÉSUMÉ

Un système de regénération du fruit de la passion *Passiflora edulis* Simis à partir du disque foliaire a été developpé. Des disques foliaires ont été cultivés sur un milieu MS modifié contenant 8,90 micromoles de BAP ou 8,90 micromoles de BAP et 2,32 de KIN. Des bourgeons apparurent après 4 semaines. Ces bourgeons pouvaient donner des racines si mis dans des milieux contenant 0,54, 2,69 et 5,37 micromoles de NAA. Des disques foliaires cultivés sur milieu contenant 11,42 micromoles de IAA, 10,74 de NAA et 1.86 micromoles de KIN ont formé des cals uniquement. Ceux cultivés sur des milieux contenant 10.74 micromoles de NAA et 2,32 micromoles de KIN ont formé des cals et des racines.

***Mots Clés*: Regénération par disques foliaires, fruit de la passion, *Passiflora edulis*, culture cellulaire.**

INTRODUCTION

The genus *Passiflora* includes more than 350 species of sub tropical vines, most of which climb by tendrils (Kuhne, 1968). Fifty to 60 of these species bear edible fruits (Martin and Nakasone, 1970). Among the cultivated species, *P. edulis* has great commercial value, especially for juice extraction (Purseglove, 1968). Two varieties of *P. edulis* are recognised, *P. edulis* Sims var. *edulis*, the purple passion fruit, and *P. edulis* Sims var. *flavicarpa* Degener, the yellow passion fruit.

In Kenya and in many other countries where passion fruit is cultivated, commercial plantings rely on seedlings (Lippman, 1978). However, vegetative propagation, mainly from rooted cuttings and grafting, is becoming increasingly important in order to propagate disease-resistant plants and clonal material of known parentage (Gilmour, 1983). Brown spot and woodiness are

the most serious diseases of this crop and can lead to decreased yields. Among the pests, root nematodes and aphids are the most important (Nattras, 1939; Samson, 1986). Woodiness, which is caused by the passion fruit woodiness virus, has no known cure, hence the need to propagate virus-free or virus-resistant varieties if higher yields have to be realised.

One possible way of obtaining improved varieties of passion fruit is by development of a plant tissue culture system for the crop. A reliable *in vitro* method for the propagation of passion fruit would have considerable benefits for the horticulture industry by allowing the rapid clonal propagation of superior-yielding, disease-resistant varieties as well as clonal multiplication of superior rootstock in areas where grafted passion fruit plants are required (Kantharajah and Dodd, 1990).

Little is known on tissue culture of passion fruit. Scorza and Janick (1979) initiated callus from leaf discs of *P. edulis* var. *flavicarpa* using a modified MS medium supplemented with 5.37 μM (1 mg l^{-1}) napthaleneacetic acid (NAA) and 4.4 μM (1 mg l^{-1}) benzylaminopurine (BAP), but no regeneration was obtained. Callus and root initials were formed when leaf discs of *P. edulis* var. *edulis* were cultured on the same medium (Scorza and Janick, 1979). Moran-Robles (1978) induced shoots via basal callus from axillary buds of var. *flavicarpa* on MS medium supplemented with 9.3 μM (2 mg l^{-1}) kinetin (KIN). Plantlet regeneration was obtained when the shoots were subcultured onto MS medium supplemented with 11.42 μM (2 mg l^{-1}) indoleacetic acid (IAA) or 9.05 μM (2 mg l^{-1}) 2,4-dichlorophenoxyacetic acid (2,4-D) or both. Kantharajah and Dodd (1990) described a technique for micro propagation of the purple passion fruit starting from seedling explants. Shoot multiplication from nodal segments of the seedlings was realised using MS medium supplemented with 8.9 μM (2 mg l^{-1}) BAP. This report describes a leaf disc regeneration system through organogenesis in *P. edulis* var. *flavicarpa*.

MATERIALS AND METHODS

Seeds and 3-month old plants were obtained from the Kenya Agricultural Research Institute (KARI) research stations at Thika for *P. edulis* var. *edulis* and Mtwapa for *P. edulis* var. *flavicarpa* and *P. edulis* var. *edulis*. During the following 12 months, when the experiments were performed, the plants were grown in soil outside.

Immature leaves excluding buds were excised from three terminal nodes of the plants. The leaves were rinsed under running tap water and surface sterilised with 70% (v/v) ethanol for 2 minutes followed by 20% (v/v) commercial bleach for 20 minutes, then rinsed three times in sterile water. Leaf discs (±1 cm^2) were cut from the sterilised leaves and cultured on a modified MS [Murashige and Skoog, 1962] medium supplemented with either auxin or cytokinin or both, as described in the results.

The medium consisted of MS salts with doubled vitamin concentration (thiamine-HCl, 0.2 mg l^{-1}; pyridoxine-HCl, 1 mg l^{-1} nicotinic acid, 1 mg l^{-1}; myo-inositol, 200 mg l^{-1}) and 4 mg l^{-1} glycine (Scorza and Janick, 1979), plus sucrose, 30 g l^{-1} and agar, 8 g l^{-1}. The cultures were incubated at 26±2°C, under a 16/8 hr light/dark cycle and 3,000 lux light intensity. Regenerated shoots were subcultured after approximately four weeks onto the modified MS medium supplemented with auxin for root induction. The plantlets obtained were transferred to jars with sterile vermiculite and watered with half-strength modified MS medium. The jars were covered with plastic foil. After one week the foil was gradually punctured until the plants were completely exposed to the environment.

RESULTS

Initiation of callus, roots or shoots. Leaf discs of var. *flavicarpa* formed callus when cultured on modified MS medium supplemented with 10.74 μM (2 mg l^{-1}) NAA, 11.42 μM (2 mg l^{-1}) IAA and 1.86 μM (0.4 mg l^{-1}) KIN. Thirty five out of 46 leaf discs (76.1%) formed callus within 22 days. Callus formation was observed from day four of culture.

In another experiment, 47 leaf discs of var. *flavicarpa* were cultured on modified MS medium supplemented with 10.74 μM (2 mg l^{-1}) NAA. Callus formation was observed from day four, while roots appeared from day 18 of culture. Only leaf discs that had formed callus formed roots. Twenty three out of 42 leaf discs (54.8%) formed callus while five (11.9%) formed roots within 28 days.

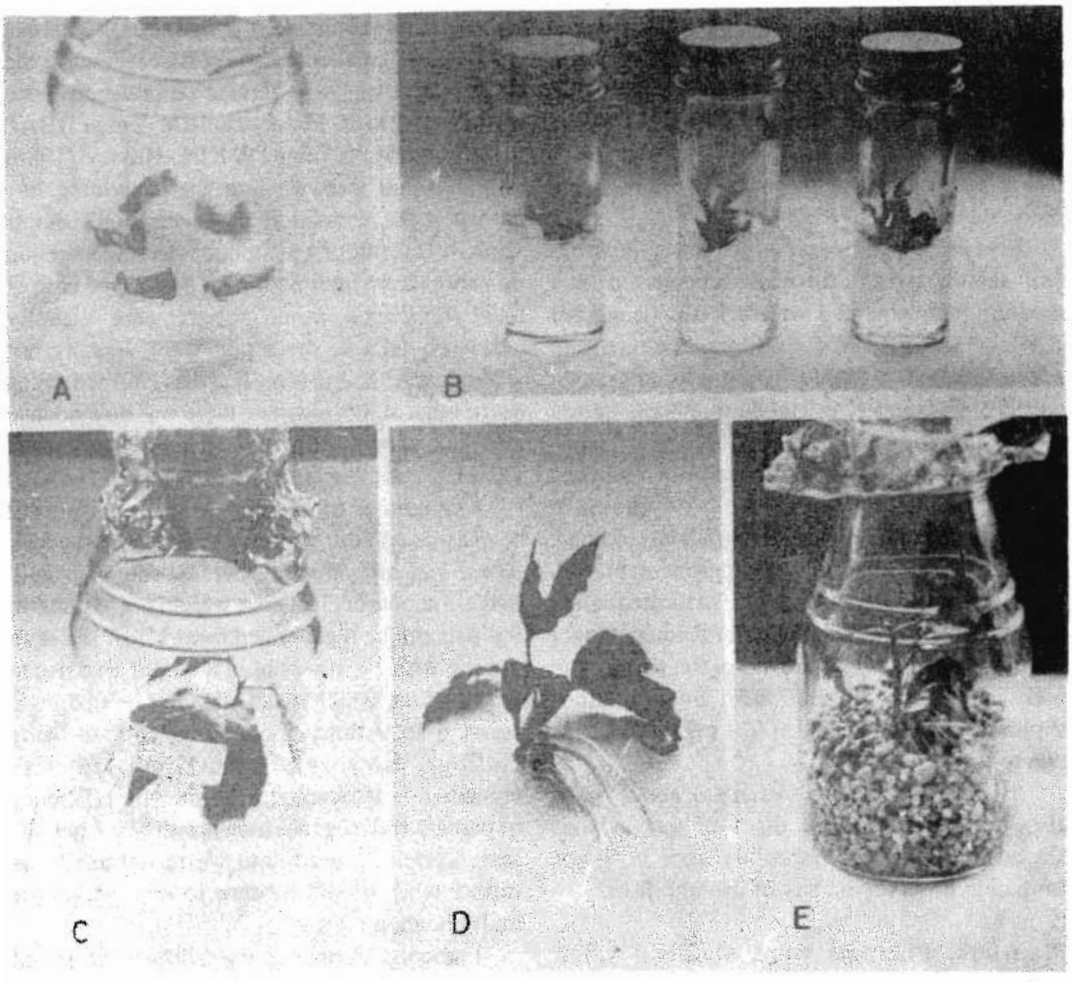

Figure 1. Leaf disc regeneration of ***Passiflora edulis* var. *flavicarpa*.**
A: 5 day old leaf discs on 8.9 µM (2 mg l^{-1}) BAP; B: 40 day old cultures showing regeneration on 8.9 µM (2 mg l^{-1}) BAP; C: 8 weeks old shoot on 2.69 µM (0.1 mg l^{-1}) NAA for root induction; D: 8 weeks old plantlet ready for transfer to vermiculite; and E: 8 weeks old plantlet after transfer to vermiculite.

In a similar experiment 50, leaf discs of var. *flavicarpa* were cultured on medium supplemented with 10.74 µM (2 mg l^{-1}) NAA and 2.32 µM (0.5 mg l^{-1}) KIN. Callus formation began to appear on day four. Twenty two leaf discs (44%) formed callus within seven days and 35 (70%) formed callus within 26 days. Again, only leaf discs that formed callus formed roots. Roots began to appear on day 13, and 23 of the cultures (65.7%) formed roots within 26 days.

When leaf discs of var. *flavicarpa* were cultured on medium supplemented with 9.05 µM (2 mg l^{-1}) 2,4-D, callus was observed on the mid vein only. There were no shoots or roots. Leaf discs cultured on medium supplemented with 9.05 µM (2 mg l^{-1}) 2,4-D and 8.9 µM (2 mg l^{-1}) BAP failed to form callus or shoots. The leaf discs only enlarged in size.

Leaf discs of var. *flavicarpa* cultured on medium supplemented with 8.9 µM (2 mg l^{-1}) BAP formed a little callus and shoots (Fig. 1A, B). In one experiment, 50 leaf discs were used and 33 of them (66%) formed callus within 21 days. However, the callus was much smaller in size

when compared with the calluses that were induced on medium supplemented with 10.74 μM (2 mg l^{-1}) NAA. Seven (14%) formed shoots within 25 days. Shoots began to appear after 18 days and were 10-15 mm long after 38 days. Most leaf discs had 2-3 shoots although up to six shoots were recorded on one leaf disc.

Leaf discs that had not formed shoots but were still healthy by day 28 were subcultured onto medium supplemented with 8.9 μM (2 mg l^{-1}) BAP. A total of 15 leaf discs were subcultured and seven of them (46.7%) formed shoots within two weeks of transfer to fresh medium. Shoots started to appear on day nine.

Similar results were obtained from leaf discs of var. *flavicarpa* cultured on medium supplemented with 8.9 μM (2 mg/l) BAP and 2.32 μM (0.5 mg l^{-1}) KIN. In one experiment, 50 leaf discs were used. Twenty four (48%) formed callus within 28 days, while 15 (30%) formed shoots within 28 days. Further growth of the shoots was realised by subculturing them onto medium supplemented with 0.44 μM (0.1 mg l^{-1}) BAP for two to four weeks.

All the experiments described above were also performed on leaf discs of var. *edulis*. However, there was no callus, root or shoot formation observed on any of the leaf discs.

Plantlets. Plantlets were obtained when regenerated shoots were induced to root on medium supplemented with 0.54 μM (0.1 mg l^{-1}), 2.69 μM, (0.5 mg l^{-1}) and 5.37 μM (1 mg l^{-1}) NAA (Fig. 1 C). Roots were observed on the shoots that were subcultured onto medium supplemented with 0.54 μM (0.1 mg l^{-1}) and 5.37 μM (1 mg l^{-1}) NAA after 13 days. Rooting occurred faster on the shoots that were about 10 mm long as opposed to those that were about 30 mm long. No roots were observed on any of the shoots subcultured onto medium supplemented with either 2.46 μM (0.5 mg l^{-1}) IBA or 5.71 μM (1 mg l^{-1}) IAA. Figure 1 D shows a plantlet ready for transfer to vermiculite while Figure 1 E shows the plantlet after transfer to vermiculite.

DISCUSSION

In the present work, callus, callus and roots as well as a little callus and shoots could be induced from leaf discs of var. *flavicarpa* by varying the concentration of auxin and cytokinin. Direct shoot regeneration without a callus phase was achieved from leaf discs of var. *flavicarpa* in the presence of 8.9 μM (2 mg l^{-1}) BAP or 8.9 μM (2 mg l^{-1}) BAP and 2.32 μM (0.5 mg l^{-1}) KIN. Hussey (1986) reported that there was no sharp dividing line between the formation of adventitious shoots directly from the parent tissue and the regeneration of shoots from intermediate callus. In the case of var. *flavicarpa*, some shoots were visually observed on leaf discs that were visually not callusing. Although some leaf discs formed callus at the edge of the mid vein, there was no evidence that the shoots were arising from the callus. Plantlets could be obtained within eight weeks.

One passion fruit leaf can easily be cut into 10 leaf discs and 3 out of 10 would regenerate shoots. Those leaf discs that regenerated shoots usually had 2-3 shoots per leaf disc, which means that 6 to 9 plants can be regenerated from 1 leaf. If a stem cutting with 1 leaf was used it would give rise to only 1 plant, which means that our procedure is about 6 to 9 times more efficient than using cuttings. However, when compared with regeneration of tobacco for instance, the efficiency of passion fruit regeneration is still low when the same system is used, and we recommend that further work should be done to improve on the multiplication rate.

In dicotyledonous species, different types of development can be obtained by treating the same cloned lines of cells with different combinations of hormones (Meins, 1986). Leaf discs of var. *flavicarpa* showed an absolute requirement for cytokinin in order to form shoots. However, leaf discs of var. *edulis* failed to form any callus, roots or shoots when various concentrations of auxin and cytokinin were used, hence the need for further work.

In var. *flavicarpa*, roots were obtained when NAA was used but no roots were obtained when IAA or indolebutyric acid (IBA) were used. Kantharajah and Dodd (1990) could not induce roots on the shoots obtained from the nodal segments of var. *edulis* using IBA. However, Muralidhar and Mehta (1982) induced rooting on the shoots obtained from leaf discs obtained from *P. alato-caerulea* using IBA. This suggests that the inability of IBA to induce rooting is unique to both varieties of *P. edulis* and needs further investigation.

Leaf discs of var. *flavicarpa* formed shoots when cultured on MS medium supplemented with 8.9 μM (2 mg l^{-1}) BAP or 8.9 μM (2 mg l^{-1}) BAP and 2.32 μM (0.5 mg l^{-1}) KIN. This suggests that the kinetin supplement is not absolutely necessary in order to initiate shoots. In *P. alato-caerulea*, leaf discs cultured on medium with 8.9 μM (2 mg l^{-1}) BAP and 2.32 μM (0.5 mg l^{-1}) KIN formed visible shoots on day 15 (Desai and Mehta, 1985). In var. *flavicarpa*, buds started to appear on the leaf discs cultured on medium with 8.9 μM (2 mg l^{-1}) BAP after 13 days. However, definite shoots with stems and leaves appeared after 20 days.

It is noteworthy that no more shoots were observed after the removal of the regenerated shoots and subsequent culture of the leaf discs onto a shoot inducing medium. However, it was interesting that some of the leaf discs that had failed to form shoots within 28 days eventually formed shoots after being transferred to the same medium. In this case, it is possible that the competent cells of such leaf discs had already reacted to the growth regulators but were not yet committed to a particular developmental fate (Halperin, 1969; Henshaw *et al.*, 1982). A second treatment with the same growth regulators therefore started the cells on a specific developmental pathway, hence the formation of the shoots.

It was not possible to reproduce Scorza and Janick's (1979) results where they reported the presence of callus and root initials on leaf discs of var. *edulis*. Failure to obtain regeneration suggests that there could be a lot of genetic variation among the purple passion fruit that has not been studied. Most of the leaf discs used started to turn brown during the sixth week and would die during the eighth week, probably due to presence of phenolics. Alternatively, the various concentrations of auxin and cytokinin that were used were unable to stimulate cell division amongst the cells that were in contact with the culture medium. It is obvious that further work needs to be done to find out why *P. edulis* var. *edulis* is recalcitrant in tissue culture.

Since it was first described by Horsch *et al.* (1985), the leaf disc regeneration system has proven to be a preferred tool for transferring genes into plants using *Agrobacterium tumefaciens*-based vector systems. Preliminary experiments in our laboratory show that *Passiflora edulis* is susceptible to *Agrobacterium* infection as wild-type tumours could be induced on *in vivo* grown *P. edulis* var. *edulis* plants. It is hoped that the leaf disc regeneration system described here will prove to be useful for isolating transgenic passion fruit plants.

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