Direct Shoot Regeneration from Strawberry Leaf Disks

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Abstract. An efficient and reproducible method of direct shoot regeneration from leaf disks in strawberry (\textit{Fragaria \times ananassa} cv. Redcoat) has been developed. The influence of hormone concentration, light intensity, orientation of leaf disk, and age of explant source on shoot regeneration was examined. Regeneration of leaf disks reached 94\%, with an average of 13 shoots per leaf disk, within 8 weeks when MS salts and B-5 vitamins medium supplemented with 10 $\mu$M each of BA and IAA were used. The adventitious shoot meristems initially arose from epidermal or subepidermal cells at the periphery of the leaf disks and later from surface cells of the newly regenerated meristems. Shoot regeneration did not depend on light, but low light intensity (12.5 $\mu$mol·s\(^{-1}·m^{-2}\)) greatly enhanced regeneration. The leaf disks obtained from 30-day-old greenhouse plants and from young runner plants produced shoots at higher frequency than those obtained from 1-year-old plants. Regeneration frequency was higher when the adaxial surface of leaf disks was kept in contact with the medium surface. Shoot regeneration also occurred in nine other genotypes at varying frequencies, but with an intervening short callus phase, except in 'Veestar'. The technique has potential application for rapid propagation and genetic manipulation of strawberries.

Tissue culture studies in strawberry primarily have been confined to meristem culture for virus elimination, vegetative propagation, and cryopreservation of germplasm (Boxus et al., 1977; Kartha et al., 1980). However, it recently has been demonstrated that plant regeneration is also possible from both primary and subcultured callus induced on immature leaves (Nehra et al., 1988; Jones et al., 1988). Shoot regeneration from callus is not only slow, but it also induces somaclonal variation (Evans and Bravo, 1986), which is undesirable in propagation, germplasm conservation, and production of transgenic plants. The introduction of foreign genes mediated by \textit{Agrobacterium tumefaciens} has now become possible in diverse plant species. The major emphasis, however, thus far has been on developing transformation protocols for model crops, such as petunia and tobacco (Horsch et al., 1985) and recently for tomato (McCormick et al., 1986) and potato (Sheerman and Bevan, 1988). Efforts have also been made to transform strawberry using similar protocols (Jelenkovic et al., 1986), but progress has been slow due to lack of an efficient regeneration system from leaf disks. We now report the results of research aimed at developing a rapid, high-frequency shoot regeneration technique from leaf disks in 'Redcoat' strawberry. Such a regeneration system should facilitate micropropagation and genetic engineering of strawberry.

Materials and Methods

\textit{Plant material.} Virus-free runner plants of seven June-bearing cultivars ('Redcoat', 'Veestar', 'Bounty', 'Kent', 'Michigan', 'Glooscap', and 'Honeoye') were obtained from Apple Berry Farms, Berwick, Nova Scotia, Canada. Three day-neutral cultivars, Hecker, Fern, and Selva were obtained from Norcal Nursery, Red Bluff, Calif. Ten plants of each cultivar were grown in a greenhouse under 16-hr photoperiod and at 26 ± 2$^\circ$C to provide explants. All plants were fertilized weekly at 0.8 g liter\(^{-1}\) with 20–20–20 soluble greenhouse fertilizer containing micronutrients.

\textit{Explant preparation and sterilization.} Young, folded leaves from the crowns of mother and daughter plants were collected and briefly washed in running tap water. The leaflets were separated and then unfolded by cutting both the midvein and then the blade horizontally at the base (see Fig. 2a). The leaf pieces thus separated were surface-sterilized in 20% Javex bleach [1.2% sodium hypochlorite (v/v)] with two drops of Tween 80 for 4 to 5 min, followed by four rinses in sterile double-distilled water. Leaf disks, 3 mm in diameter, were cut from the middle portion of the leaflet segments.

\textit{Culture medium.} The culture medium (MS) was Murashige and Skoog (1962) inorganic salts, 30 g sucrose/liter, 8 g Difco Bacto-agar/liter, and B-5 vitamins (Gamborg et al., 1968). The pH of the medium was adjusted to 5.8 with KOH or HCl. BA was added to the medium before autoclaving, while IAA was filter-sterilized (0.45 $\mu$M) and added post-autoclaving. Media were autoclaved for 30 min. at 125$^\circ$C and dispensed as 25-ml aliquots into 110-ml glass jars.

\textit{Effect of hormone concentration and light intensity.} The effect of BA and IAA combined at 0, 1, 5, 10, or 20 $\mu$M each on shoot regeneration was tested by incubating the cultures at 26$^\circ$C and a 16-hr photoperiod of 12.5 $\mu$mol·s\(^{-1}·m^{-2}\). The response of light intensity levels in the range of 0 to 125 $\mu$mol·s\(^{-1}·m^{-2}\) on shoot regeneration was studied by culturing the leaf disks on MS + 10 $\mu$M each of BA and IAA. The light sources were four Sylvania cool-white fluorescent tubes and two incandescent bulbs. Control cultures were incubated in the dark.

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The leaf disks for both experiments were inverted within 5 days to bring the adaxial surface in contact with the medium.

Effect of leaf disk orientation and plant age. Explants prepared from the tips of young runner plants were used to study the effect of orientation of leaf disks on shoot regeneration. In another experiment, explants obtained from the tips of young runner plants and from the crowns of 1-month-old greenhouse plants were compared for regeneration capacity with those obtained from crowns of 1-year-old greenhouse plants. The leaf disks in this experiment were placed with their adaxial surface in contact with the medium. Cultures for both experiments were incubated on MS + 10 μM each of BA and IAA at 26°C and a 16-hr photoperiod of 12.5 μmol·s⁻¹·m⁻².

Effect of genotype. Seven June-bearing and three day-neutral cultivars were tested for their regeneration capacity from leaf disks under culture conditions standardized for 'Redcoat'. To achieve uniformity of plant age, leaf explants were taken from 1-year-old greenhouse plants of all cultivars, except 'Fern', where 2-week-old plants were used.

Experimental design, data collection and analyses. A completely randomized design was used for all experiments. Each treatment consisted of six replications (jars) with four leaf disks in each jar. All the experiments were run twice. The final data are reported as an average of 12 replications with 48 leaf disks in each treatment. The cultures were scored after 4 and 8 weeks for percentage of leaf disks that regenerated shoots and for total number of shoots per leaf disk. The number of shoots per leaf disk was expressed as a ratio of total number of shoots/number of leaf disks producing shoots. This ratio presents a more accurate expression of average number of shoots per leaf disk than unadjusted data because many disks did not produce shoots in certain treatments. Percentage data were transformed to arcsin of square root and other data to square root before analysis of variance was conducted. Mean separation was performed by using a paired t test to compare two treatments or by Tukey's HSD (P = 0.05) for more than two treatments.

Histology. Leaf disks were fixed in 3% glutaraldehyde, dehydrated through an ethanol series at 0°C and embedded in historesin. The tissues were sectioned at a thickness of 3 μm and stained with 1% toluidine blue (w/v) in citrate phosphate buffer, pH 4.4, for 3 min (Fowke, 1982).

Results

Effect of hormone concentration. Various combinations of cytokinin (BA) and auxins (IAA, NAA, 2,4-D) were tested in a preliminary experiment. Only BA and IAA each at 5 μM induced direct shoot regeneration in 'Redcoat'. The subsequent experiment indicated that 10 μM each of BA and IAA was optimum for shoot regeneration (Fig. 1). Shoot regeneration also occurred at lower and higher concentrations, but at much lower frequencies. The regeneration frequency and number of shoots per leaf disk were higher after 8 than after 4 weeks in culture at all concentrations tested. However, a maximum number of shoots per leaf disk (11) was still observed at 10 μM each of BA + IAA.

Shoot regeneration began in 2 weeks and multiple shoots developed all along the periphery of the leaf disks at 5 and 10 μM each of BA + IAA after 4 to 6 weeks (Fig. 2b), however, at 1 μM each of BA and IAA, only a few single shoots emerged from leaf disks (Fig. 2c), which later elongated rapidly and developed roots. Only callus was observed at 20 μM, the highest concentration we used (Fig. 2d). Shoots rarely developed from these cultures even after 8 weeks.

Histological studies revealed that the adventitious shoot meristems were initiated directly from either subepidermal or epidermal cells at the periphery of the leaf disk (Fig. 2e) after 4 to 5 days in culture; more shoot meristems were observed after 11 days in culture (Fig. 2f). Secondary shoot meristems developed from single cells at the surface of primary shoot meristems at first, but later the number of shoots increased by axillary bud development.

Effect of light intensity. Slender etiolated shoots arose from leaf disks at low frequency even in the absence of light (Fig. 3). However, the optimum level of light intensity for shoot initiation and proliferation was 12.5 μmol·s⁻¹·m⁻². The regeneration frequency as well as number of shoots per leaf disk decreased considerably at 25 μmol·s⁻¹·m⁻² while, at 62.5 μmol·s⁻¹·m⁻² and above, the leaf disks expanded rapidly, but eventually turned brown at the periphery (Fig. 2g) and did not regenerate.

Effect of leaf disk orientation. The leaf disks began to expand immediately after their placement in culture and subsequently curled either toward or away from the medium surface. Examination of the curled disks revealed that, if leaf disks were placed with the adaxial surface towards the medium, the cut margins curled upward and vice versa. In this experiment, the adaxial and abaxial surface of leaf disks was identified under the microscope before explanting. A significantly higher percentage of leaf disks regenerated and more shoots per leaf disk were observed when the adaxial surface of the leaf disk was placed in contact with the medium (Table 1). This orientation...
Shoot regeneration in strawberry leaf disks. (a) Leaf disks prepared from young unfolded leaflets. (b) Multiple shoot development at the periphery of a leaf disk on MS medium with 10 μM each of BA and IAA. (c) Single shoot initiated at 1 μM each of BA and IAA. (d) Callus formation at the periphery of a leaf disk at 20 μM each of BA and IAA. (e) Leaf cross-section showing initiation of a shoot meristem from a sub-epidermal or an epidermal cell after 4 to 5 days in culture. × 400. (f) Leaf cross-section showing development of primary and secondary shoot meristems after 11 days in culture. × 160. (g) A typical expanded leaf disk that did not regenerate shoots at 62.5 μmol·s⁻¹·m⁻² of light intensity. (h) Emergence of adventitious shoots from callus in ‘Bounty’.

also resulted in a 50% increase in leaf disks producing shoots after 4 weeks of culture.

Plant age and regeneration. The percentage of leaf disks forming shoots was significantly higher after 4 weeks in culture when the explants were obtained from young runner plants or 1-month-old greenhouse plants than from 1-year-old plants (Table 2). Also, explants from young runner plants yielded a higher number of shoots per leaf disk. No significant differences for percentage of leaf disk regeneration and number of shoots per leaf disk were observed after 8 weeks in culture (72% to 94% and 11 to 13, respectively).

Genotypic response to regeneration. Genotypic differences in the ability of strawberry leaf disks to regenerate shoots and average number of shoots per leaf disk were observed in response to the culture conditions for standardized ‘Redcoat’. All cultivars regenerated, although at much lower frequency than ‘Redcoat’ (Table 3). The second-best response was observed in ‘Veestar’, which is a reciprocal cross of ‘Redcoat’ parents. Only these two cultivars showed direct shoot regeneration without an intermediate callus phase, whereas in other genotypes, regeneration occurred via intermediary callus around the periphery of the leaf disks (Fig. 2h). Friable, pinkish brown callus growth was observed in ‘Bounty’, ‘Glooscap’, and ‘Hecker’, whereas the rest of the cultivars formed green compact calli before shoot regeneration.

Discussion

Hormonal regulation of auxin and cytokinin balance has long been recognized as a key factor in the control of cell division and organogenesis in most dicot plants (Murashige, 1977). Our research demonstrated that an equal ratio of exogenously applied BA and IAA, 10 μM each, was essential for direct, high-frequency shoot regeneration from leaf disks of ‘Redcoat’ strawberry. The difference in regeneration capacity and mode of regeneration at concentrations higher and lower than optimum may be explained on the basis of variation in the endogenous levels of these growth hormones in leaf tissues. Similar observations regarding the role of endogenous hormone levels in determining the shoot-forming capacity of tomato leaf disks have been reported (Karthia et al., 1976; Frankenberger et al., 1981). A recent study (Elliott et al., 1987) has also demonstrated that a critical endogenous level of IAA has to be attained before cell division and organogenesis could occur in suspension cultures. Apparently, shoot regeneration from leaf disks was not dependent on light, which is contrary to most tissue culture studies, where light has been found to be essential. However, we found that low light intensity levels considerably enhanced shoot regeneration. These results are consistent with a recent study by Parthier et al. (1987) on hormone and light action in organ differentiation. Their study also indicated that growth hormones alone were capable of stimulating organ development, but a low
Table 2. Effect of plant age on shoot regeneration from leaf disks of 'Redcoat' strawberry.

<table>
<thead>
<tr>
<th>Age of donor plants</th>
<th>Leaf disks forming shoots (%)</th>
<th>No. shoots per leaf disk†</th>
</tr>
</thead>
<tbody>
<tr>
<td>One year</td>
<td>50 a</td>
<td>3.3 a</td>
</tr>
<tr>
<td>Young runners</td>
<td>78 b</td>
<td>7.4 b</td>
</tr>
<tr>
<td>One month</td>
<td>83 b</td>
<td>4.0 a</td>
</tr>
</tbody>
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*Means separated within column by Tukey's HSD (P = 0.05) on transformed data. Original means are presented.

Table 3. Shoot regeneration (±SE) from leaf disks of 10 strawberry cultivars.

<table>
<thead>
<tr>
<th>Cultivar</th>
<th>4 weeks</th>
<th>8 weeks</th>
<th>No. shoots per leaf disk†</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>June-bearing</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Redcoat</td>
<td>58 ± 10</td>
<td>79 ± 7.7</td>
<td>4.5 ± 0.5 11 ± 2.5</td>
</tr>
<tr>
<td>Veestar</td>
<td>17 ± 8.3</td>
<td>42 ± 12</td>
<td>1.7 ± 0.7 5.6 ± 2.0</td>
</tr>
<tr>
<td>Kent</td>
<td>17 ± 5.3</td>
<td>29 ± 10</td>
<td>1.3 ± 0.3 3.3 ± 0.5</td>
</tr>
<tr>
<td>Bounty</td>
<td>17 ± 8.3</td>
<td>25 ± 11</td>
<td>1.7 ± 0.7 2.4 ± 0.6</td>
</tr>
<tr>
<td>Glooscap</td>
<td>12 ± 5.6</td>
<td>21 ± 4</td>
<td>1.0 ± 0.0 3.6 ± 1.4</td>
</tr>
<tr>
<td>Micmac</td>
<td>12 ± 5.6</td>
<td>17 ± 5.3</td>
<td>3.7 ± 0.9 4.7 ± 1.3</td>
</tr>
<tr>
<td>Honeoye</td>
<td>4.2 ± 4.2</td>
<td>4.2 ± 4.2</td>
<td>1.0 ± 0.0 5.0 ± 0.0</td>
</tr>
<tr>
<td>Day-neutral</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fern</td>
<td>21 ± 12</td>
<td>25 ± 13</td>
<td>1.0 ± 0.0 2.4 ± 0.5</td>
</tr>
<tr>
<td>Selva</td>
<td>15 ± 6.1</td>
<td>25 ± 7.9</td>
<td>1.3 ± 0.3 3.1 ± 0.7</td>
</tr>
<tr>
<td>Hecker</td>
<td>12 ± 8.5</td>
<td>25 ± 11</td>
<td>1.0 ± 0.0 1.5 ± 0.3</td>
</tr>
</tbody>
</table>

*Total no. of shoots/no. of disks forming shoots.

level of illumination greatly enhanced the process. Increased shoot formation with a slight increase in light intensity levels has also been reported in other plant species (Hughes, 1981). The higher light intensities used in our study were detrimental to shoot regeneration, possibly due to photooxidation of IAA (Fridborg and Eriksson, 1975).

Histological examination of cultured leaf disks indicated that the adventitious shoots developed directly from either a sub-epidermal or an epidermal cell at the periphery of the leaf disks. Periclinal division of a single epidermal or sub-epidermal cell followed by anticlinal and periclinal divisions suggests a single cell origin. The cell division pattern (Fig. 2e) is not unlike that published by Broertjes (1968) in support of single cell shoot origin of Saintpaulia. Further proliferation of shoots was initially by formation of proembryo-like structures from single cells at the surface of primary shoot meristems and later by axillary bud development. This pattern of shoot development may help in maintaining the genetic stability among regenerants.

Orientation of leaf disks on the medium surface and age of the explant donor were also important in obtaining high-frequency regeneration from leaf disks. Regeneration frequency improved considerably when the adaxial rather than the abaxial surface of the leaf disk was placed in contact with the medium. This difference in regeneration potential due to leaf disk orientation may have resulted from polar transport of IAA into the cells at the periphery of the leaf disks. Similar polarity effects were also observed by Leshem et al. (1982) in bulb scale explants.

Short-term (4 weeks) regeneration capacity was optimum when the explants were obtained from young runners. The older plants were fruiting when the explants were excised. Thus, the nutrients and growth hormones may have been diverted to the developing fruits, resulting in redistribution of endogenous levels of hormones. This change could be one possibility for a poor initial response and reduced regeneration frequency in explants taken from mature plants. The effect of age of explant source on regeneration has been reported in trees (Cheng, 1975) and herbaceous plants (Tanimoto and Harada, 1980).

In this study, we have also demonstrated that genotypic variation exists for shoot regeneration from leaf disks in strawberry. Regeneration occurred in all genotypes at varying frequencies—high frequency, direct shoot regeneration was specific to 'Redcoat' and 'Veestar'. The phenomenon of genotypic differences
in shoot regeneration capacity has been reported earlier in strawberry (Jones et al., 1988) and also in tomato (Kurtz and Lineberger, 1983; Tan et al., 1987) and other plant species (Green, 1977; Jarret al., 1980).

A quick, efficient, and reproducible direct shoot regeneration protocol from leaf disks has been developed for ‘Redcoat’ strawberry. The method avoids an intermediate callus phase and, therefore, may reduce the risk of producing somaclonal variation. Shoot regeneration began after 2 weeks in culture; >80% of the leaf disks regenerated within 4 weeks. The regenerated shoots were easily rooted on hormone-free medium and transplanted to a greenhouse with 100% survival (data not shown). The regenerants taken to maturity in the greenhouse were found to be typical of ‘Redcoat’ in growth, leaf morphology, and fruit characters. Once the genetic stability of these plants is confirmed, the method may be promising for rapid plant multiplication and development of a protocol for genetic transformation of strawberry using *A. tumefaciens*.

**Literature Cited**


