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Full Length Research Paper

High frequency shoot organogenesis in juvenile leaf of *Duchesnea indica* (Andr.) Focke

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An *in vitro* cultivation protocol was developed for *Duchesnea indica* (Andr.) Focke. It is an important medicinal plant in China. Adventitious shoot induction was most successful by using young leaves as explants for propagation on Murashige and Skoog (1962) medium (MS) supplemented with 8.5 μM N_6-benzylaminopurine (BA) and 2.0 μM Indole-3-acetic acid (IAA). For continuous subculture, α-Naphthaleneacetic acid (NAA) and BA (μM) at a ratio of 2.5:4.5 had the best regeneration potential producing approximately six plantlets per leaf explant. The highest frequency of induction adventitious roots was 91% on the 2/3-strength MS medium with butyric acid (IBA) 2.0 μM and IAA 2.5 μM. The results show that the basic medium played a determinative role in initiating the adventitious roots. This propagation regime has the capacity for producing 1500 to 2000 plants from one young leaf after three months long subculture cycles, making it highly attractive for implementation as an *in vitro* conservation strategy. The micro-propagated plants were easily acclimatized (85%) within a month after rooting *in vitro* and planted *ex vitro* with soil: plant ash: compound fertilizer (30:2:1; weight.) mixture.

Key words: Medicinal herbs, *Duchesnea indica* (Andr.) Focke, micropropagation.

INTRODUCTION

Duchesnea indica (Andr.) Focke belongs to the Rosaceae family, plants perennial, stoloniferous (producing roots at the nodes); stolones thin, up to ca. 100 cm long, covered with scattered pilose to strigose hairs; leaves alternate, stipulate, trifoliate, small, 3-5×4-6 cm, normally 3-lobed but rarely with 4-5 lobes; leaflets ovate, serrate, strigose above and below (especially on veins); stipules oblong-lanceolate; inflorescence usually erect, peduncles up to 10 cm long, usually ending to one flower; outer sepals foliaceous, 3-5-toothed or lobed at apex; inner sepals as long as the outer or almost so, ovate-lanceolate and apiculate to aristate, ciliolate at margins; with five 3-lobed bracts alternating with the sepals. Bracts ca. 6 mm long and broad in flowering; petals 5, ovate, yellow, 4-5*7-8 mm; fruit receptacle red, conic to ovate, with short stalk, edible, much like a strawberry but tasteless. Despite strawberry, the fruit has a rough surface with scattered, raised achenes growing in mountain slopes, meadows, river banks, wet places; below 1800 m. *D. indica* occurs in China, Afghanistan, Bhutan, India, Indonesia, Japan, Korea, Nepal, Sikkim; naturalized in Africa, Europe, and North America (Zare et al., 2007). It flowers from June to August and fruits from August to October. Due to its ecological preferences (Jackowiak, 1992; Lauber and Wagner, 2001), species mostly inhabits relatively moist, nitrophilous and shaded habitats. The chromosomal number of *D. indica* is 2n = 84 (Naruhashi et al., 1986; Naruhashi and Takano, 1987; Zhao et al., 2008; Naruhashi and Iwatsubo, 1991; Xu et al., 1992).

D. indica possesses a variety of biological activities,

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Abbreviations: **BA,** N_6-Benzylaminopurine; **IAA,** indole-3-acetic acid; **NAA,** -naphthaleneacetic acid; **IBA,** indole-3-butyric acid; **MS,** Murashige and Skoog (1962) medium; **2,4-D,** 2,4 dichlorophenoxyacetic acid; **PGR,** plant growth regulator; **LSD,** least significant difference.

including cytotoxic, antibiotic, antioxidative, and anti-inammatory activities (Lee and Kim, 1986; Kim et al., 2002; Kim et al., 2007). Several chemical compounds (for example, triterpenes, triterpene glycodides, flavonoid glycodides, sterols) have been isolated from *D. indica* (Lee and Yang, 1994; Qiao et al., 2009; Peng et al., 2008). Some researches suggest that the methanolic extract of *D. indica* (DIM) directly acted on free radicals (Hu et al., 2009). *D. indica* (Andrews) Focke extract showed strong anti-inflammatory activity, in particular suppressing pro-inflammatory cytokines and mediator by blocking NF-kB activation (Zhao et al., 2008). *D. indica* (Andr.) Focke has been documented as anti-inflammatory. It is often used for cancer therapy alone or as a main ingredient in the formulas with traditional reputed benefits for the treatment of cancer in China and Japan. The aqueous extract of *D. indica* was reported to have antiproliferative activity *in vitro* against many different types of cancer cells (Shoe et al., 2005; Peng et al., 2008).

To meet increasing commercial demands, large quantities of medical plants are desirable for the global pharmaceutical industries. *D. indica* (Andr.) Focke seeds also have the problem of low germination rate, low orderliness and long seedling time. The rapidness of tissue culture techniques can be advantageous for the continuous provision of a plantlet stock for cultivation and may further compliment breeding programmes. We investigated the most suitable *in vitro* propagation protocol for the production of *D. indica* (Andr.) Focke but to our knowledge, there are no reports on the micropropagation of *D. indica* (Andr.) Focke.

MATERIALS AND METHODS

Leaves of *D. indica* (Andr.) Focke were collected seasonally from wild populations growing within the southwest region. The leaves were incubated in 75% (v/v) ethanol for 1 min followed by surface decontamination in 0.1% (w/v) $HgCl_2$ for 8 min. Afterwards, the leaves were washed four times (5 min) with sterile distilled water prior to placing onto tissue culture medium (pH 5.8) with a Murashige and Skoog (MS) (1962) salts solidification with agar [0.7% (w/v) Agar-agar, Associated Sangon Biotech (Shanghai) Co. Ltd. (China)]. After decontamination, some of the leaves were placed onto 0.8% water-agar (pH 5.8; controls). During this study, all media were autoclaved at 121°C and 101 kPa for 20 min after adjustment of the pH to 5.8 with 1 M NaOH. When cool, 25 ml of the medium was poured into 50 ml conical flask. The leaves were transferred to a 26 to 28°C growth room with 16 h light illumination (250 mol $m^{-2}s^{-1}$ photosynthetic photon densities) and 8 h dark. The light was provided by ±cool-whiteq' uorescent tubes (40W/220V×6). Leaves of *D. indica* (Andr.) Focke were monitored every two days.

Callus induction

Primary leaf tissue was obtained from wild seedlings that were 30 to 50 days old. The explants were cut and transferred under laminar conditions to callus induction medium [25 ml MS with 0.15 gl^{-l} myo-inositol, 30 gl^{-l} sucrose and 1% (w/v) agar (pH 5.8)]. Auxins and cytokinins were added individually to the different media. The auxins used were -Naphthaleneacetic acid (NAA) (1.0, 2.0 or 3.0 M); Indole-3-acetic acid (IAA) (1.0, 2.0 or 3.0 M) or 2,4 dichlorophenoxyacetic acid (2,4-D) (1.0, 2.0 or 3.0 M), whereas N_6-benzylaminopurine (BA) (2.0, 4.5 or 8.5 M) was the cytokinins investigated for organogenesis.

The plant segments were cultured on the induction medium in conical flasks (50 ml) capped with lids that had a 10 mm vent covered with a 0.22 lm polypropylene membrane. Flasks were returned to the continuous light growth room (200 mol $m^{-2}s^{-1}$ photosynthetic photon density) with the temperature ranging from 26 to 28°C, with relative humidity of 70 to 80%. These culture conditions were used for all subsequent experiments. The survival ratio and fresh weights of callus were weighed after 30 days in culture, mean number of shoot per callus were calculated after 70 d, and shoot length were measured after 90 days. As all tissue culture experiments were repeated thrice, the combinations that are represented in Table 1 were the callus induction experiment.

Multiplication of plantlets

In order to establish a system which could be utilized for continuous micro-plant production and sub-culturing, combinations of NAA, butyric acid (IBA) or IAA and BA were tested for their ability to multiply and elongate *in vitro* shoots of *D. indica* (Andr.) Focke. After 30 days, the calli was transferred to regeneration media (NAA and BA combinations, IAA and BA combinations or IBA and BA combinations) (Table 2). Observations were recorded on percentage of shoot production, mean number of shoot per callus and shoot length.

Rooting

Plantlets on the maintenance medium [3.0:4.5; IAA: BA (M, respectively)], were rooted after continuous culture (3 to 5 cycles). The plantlets (5 to10 cm) were placed on 1/2-strength MS medium, 2/3-strength MS medium or MS medium. These medium contained also: 30 g l^{-1} sucrose, and 0.1 g l^{-1} myo-inositol. The composition in 1/2 MS was similar to MS except for 1/2 large elements, and so was 2/3 MS.

Plantlets were rooted over a four-week period in 1/2 MS, 2/3 MS or MS, as a stimulus for rooting, IBA and IAA was included in the media; the concentration of IBA was 2.0, 3.0 or 4.0 M, and the concentration of IAA was 2.5, 5.5 or 8.5 M. The controls lacked this rooting additive. When plants had rooted, extraction of plants was done with care in order not to break the roots. Observations were recorded on percentage of root production, mean number of roots per plantlet and root length.

Acclimatization

In the spring of 2010, all plantlets from the rooting treatments were prepared for acclimatization by gently washing off the culture medium thoroughly, using sterilized distilled water prior to transfer to transplantation substrate. Two different transplantation substrates were tested. Only rooted plantlets were transferred to the culture vessels containing mixture of soil: plant ash: compound fertilizer (30:2:1; weight). The transplantation substrate was autoclaved before use and one plantlet was placed per culture vessel (200×90 mm). To maintain the same composition of macro- and micro-nutrients prior to acclimatization, liquid ½ MS (lacking sucrose and myo-inositol) was used to wet the acclimatization substrate. The culture vessels containing the plantlets were all sealed with lids that had a 10 mm vent covered with a 0.22 m polypropylene membrane.

Table 1. The data of callus induction experiment.

Combination of IAA and BA (µM)	fresh weight of callus (mg)	Survival ratio of callus (%)	Mean number of shoot per callus	Shoot length (cm)
3.0 IAA +2.0 BA	52.5±3.78[b]	75±3.55[c]	4.5±1.52[a]	4.50±1.25[a]
1.0 IAA +4.5 BA	73.5±4.11[a]	87±4.43[b]	4.0±1.44[b]	3.89±1.35[b]
2.0 IAA +8.5 BA	78.4±5.23[a]	89±6.21[a]	4.5±2.20[a]	4.76±2.23[a]
3.0 NAA +2.0 BA	80.5±5.13[a]	88±4.37[a]	4.5±1.31[a]	3.87±1.46[b]
1.0 NAA +4.5 BA	65.5±3.77[b]	94±2.71[a]	4.0±1.65[b]	4.07±2.32[b]
2.0 NAA +8.5 BA	70.3±4.25[b]	85±3.42[b]	4.0±2.24[b]	4.32±2.55[b]
3.0 2,4-D +2.0 BA	77.5±4.53[a]	86±2.52[b]	3.5±0.84[c]	3.5±0.92[c]
1.0 2,4-D +4.5 BA	72.6±5.13[a]	82±4.37[c]	3.5±1.31[c]	3.7±1.46[c]
2.0 2,4-D +8.5 BA	79.2±3.12[a]	80±3.44[c]	4.0±2.21[b]	4.0±2.12[b]

The survival ratio and fresh weight s of callus 30 days in culture, mean number of shoot per callus were calculated after 70 days, and shoot length were measured after 90 days. Data indicate mean ± standard error and treatments denoted by the same letter in a column were not different (P m0.05) using the LSD test. Ten replicates were used per treatment and experiments were repeated thrice.

These were transferred to a thermostatically controlled glasshouse with natural sunlight and the culture vessels containing the plantlets were exposed to a photosynthetic photon flux density (PPFD) of range of 800 to1000 mol m^{-2} s^{-1} (midday irradiance). The glasshouse thermostat was set to regulate the minimum temperature at 18°C and the maximum temperature at 28°C. The acclimatization period started on August 20 and from then onwards, the glasshouse conditions were kept the same. On September 10, plantlets were transplanted from the culture vessels to pots and the number of acclimatized plantlets was recorded on October 10. During this time-period, the natural day-length increased from 10 to 12 h with outdoor temperatures ranging from 14 to 24°C. The lids of the culture vessels were loosened over the acclimatization period until they were eventually completely removed (August 20 to September 10). This reduced the relative humidity from 90% to about 70%. For each transplantation substrate, 15 micro-plants were transferred from the rooting medium to soil and the acclimatization experiments were repeated thrice.

Ex vitro growth

Plantlets were allowed to grow for one week without lids prior to transfer to pots (10 cm) , lled with soil: plant ash: compound fertilizer (30:2:1; wt.) on September 10. On this day, the number of potted plants was recorded. Those plantlets acclimatized in vermiculite inside culture vessels were transplanted to pots with the same transplantation substrate. Once in pots, plants were watered by hand two to three times a week. When a white powdery fungus was noted on the shoots, they were sprayed with broad-spectrum fungicides which also act against powdery mildews. Plants were monitored regularly. The tally of successfully acclimatized plants and the length of the propagules were recorded one month after been transferred to pots on October 10.

Statistical analysis

For culture induction, multiplication and rooting experiments, one explant was placed per flask and each flask represented a replicate. Three replicates were used per treatment. Experiments were based on a completely randomized design. All quantitative data were subjected to standard analysis of variance using the General linear model procedure generated by the SPSS14.0 program (Tables 1 to 3). The least-squares means (LSM) and least significant difference (LSD) values were calculated at a 5% signi, cant level. The test controls the Type I comparison-wise error rate. Otherwise, data from all other experiments were subjected to the M-L ^{2} Chi square analysis using Statistical release 7 (Statsoft Inc. 2005) to test whether the observed differences were or not at a 5% level.

RESULTS AND DISCUSSION

Callus induction

The survival ratio and fresh weights of callus 30 days in culture, mean number of shoot per callus were calculated after 70 days, and shoot length were measured after 90 days. Callus production (Figure 1B) was proli, c for most plant growth regulator (PGR) treatments (Table 1), fresh weight of callus at a range of 52.5 to 79.0, survival ratio of callus at a frequency of 75 to 94%, and mean number of shoot per callus from 3.5 to 4.5 length. The longest shoot was 4.76 cm in length, and the shortest shoot was 2.7 cm. Adventitious shoot regeneration (Figure 1C) was strongly in' uenced by the combination of auxin. When 2.0 µM IAA was combined with BA at 8.5 µM, callus induction differed from the other combinations tested with fresh weight of callus of 78.4 mg, survival ratio of callus of 89% and an average of 4.5 shoots produced. Indirect organogenesis increase the probability of genetic variations in culture (George and Sherrington, 1984), which may adversely affect the quality of the essential constituents produced from propagated plants.

Multiplication for continuous culture

When 2.5 µM NAA was combined with BA at 4.5 µM, shoot induction differed from the other combinations tested with an average of 6.0 shoots produced (Table 2) (P = 0.002). Although these treatments caused shoot regeneration, their effect on shoot elongation was

Table 2. Regeneration of callus, shoots and/or roots from primary leaf explants of *Duchesnea indica* (Andr.) Focke on Murashige and Skoog (1962) (MS) media supplemented with different auxin/cytokinin combinations for a 30-day period.

PGR combination (µM)	percentage of shoot production (%)	Mean number of shoot per callus	Shoot length (cm)
1.0 NAA+2.0 BA	57	5.5±1.55[a]	3.20±1.25[b]
2.5 NAA+4.5 BA	82	6.0±1.24[a]	3.49±1.35[a]
5.0 NAA+8.8 BA	53	5.2±2.25[a]	3.76±2.23[a]
1.0 IAA+2.0 BA	79	5.5±1.35[a]	2.87±1.46[b]
2.5 IAA+4.5 BA	61	5.0±1.45[b]	3.07±2.32[b]
5.0 IAA+8.5 BA	75	5.0±2.20[b]	3.32±2.55[b]
1.0 2,4-D +2.0 BA	65	4.5±0.54[c]	2.5±0.92[c]
2.5 2,4-D +4.5 BA	57	4.5±2.01[c]	2.7±1.46[c]
5.02,4-D +8.5 BA	56	5.0±2.32[b]	3.0±2.12[b]

Data collected from media that failed to induce a regeneration response or that resulted only in explant elongation after 30 days without evident callus, shoot and/or root formation within 30 days were omitted. Ten replicates were used per treatment and experiments were repeated thrice.

Table 3. Rooting of *Duchesnea indica* (Andr.) Focke plantlets on different Murashige and Skoog (1962) medium (MS) with different concentrations of butyric acid (IBA) and Indole-3-acetic acid (IAA) for 3 weeks.

Combination of basic medium and auxin (µM)	Percentage of root production (%)	Mean number of roots per plantlet	Root length (cm)
1/2MS+2.0 IBA+2.5 IAA	87	5±2.08[b]	3.05±1.38[b]
1/2MS+3.0 IBA+5.5 IAA	82	6±1.52[a]	3.44±2.40[b]
1/2MS+4.0 IBA+8.5 IAA	83	7±1.55[a]	3.01±2.32[c]
2/3MS+2.0 IBA+2.5 IAA	91	7±2.68[a]	4.05±1.35[a]
2/3MS+3.0 IBA+5.5 IAA	91	6±2.62[a]	3.24±2.41[b]
2/3MS+4.0 IBA+8.5 IAA	95	5±3.45[b]	4.01±2.35[a]
MS+2.0 IBA+2.5 IAA	95	5±2.38[b]	2.75±2.36[c]
MS+3.0 IBA+5.5 IAA	86	5±2.62[b]	3.24±1.40[b]
MS+4.0 IBA+8.5 IAA	86	6±3.85[a]	3.01±2.32[c]

Data indicate mean ± standard error and treatments denoted by the same letter in a column were not different (P m 0.05) using the LSD test. Ten replicates were used per treatment and experiments were repeated twice.

statistically similar to the other IAA and BA combinations. This may imply that for shoot regeneration, the ratio of NAA or IAA to BA is important for successful plantlet regeneration. However, the internodal elongation does not appear to be dependent on the auxin:cytokinin ratio as the length of those on cytokinin alone was similar to those on the combined PGRs.

There were no signi, cant differences with respect to the multiplication and/or elongation potential when the medium contained NAA or IAA and BA at a ratio of 2.5:4.5(M). As a result, the former PGR combination is now routinely utilized in our laboratories for continuous culture of *D. indica* (Andr.) Focke. On average, 4 to 5 nodes became available from each plantlet (3.0 to 4.0 cm) at the time of subculture. With an average of four plantlets regeneration per node, after three to four weeks cycles, approximately 1500 to 2000 plants may be produced from one young leaf, thus validating the use of this continuous culture regime.

In vitro rooting and *ex vitro* transfer

For preliminary experiments, plantlets were left to root on medium that lacked IBA or IAA for a month. This treatment did not appear to be for rooting. *D. indica* (Andr.) Focke was able to root spontaneously on multiplication medium with 3 to 7 roots produced per plantlet (Table 3, Figure 1D). Even so, root induction could be improved by incubating plantlets in 2/3 MS medium containing IBA and IAA at a ratio of 2.0:2.5 M. This treatment produced seven roots per plantlet (Table

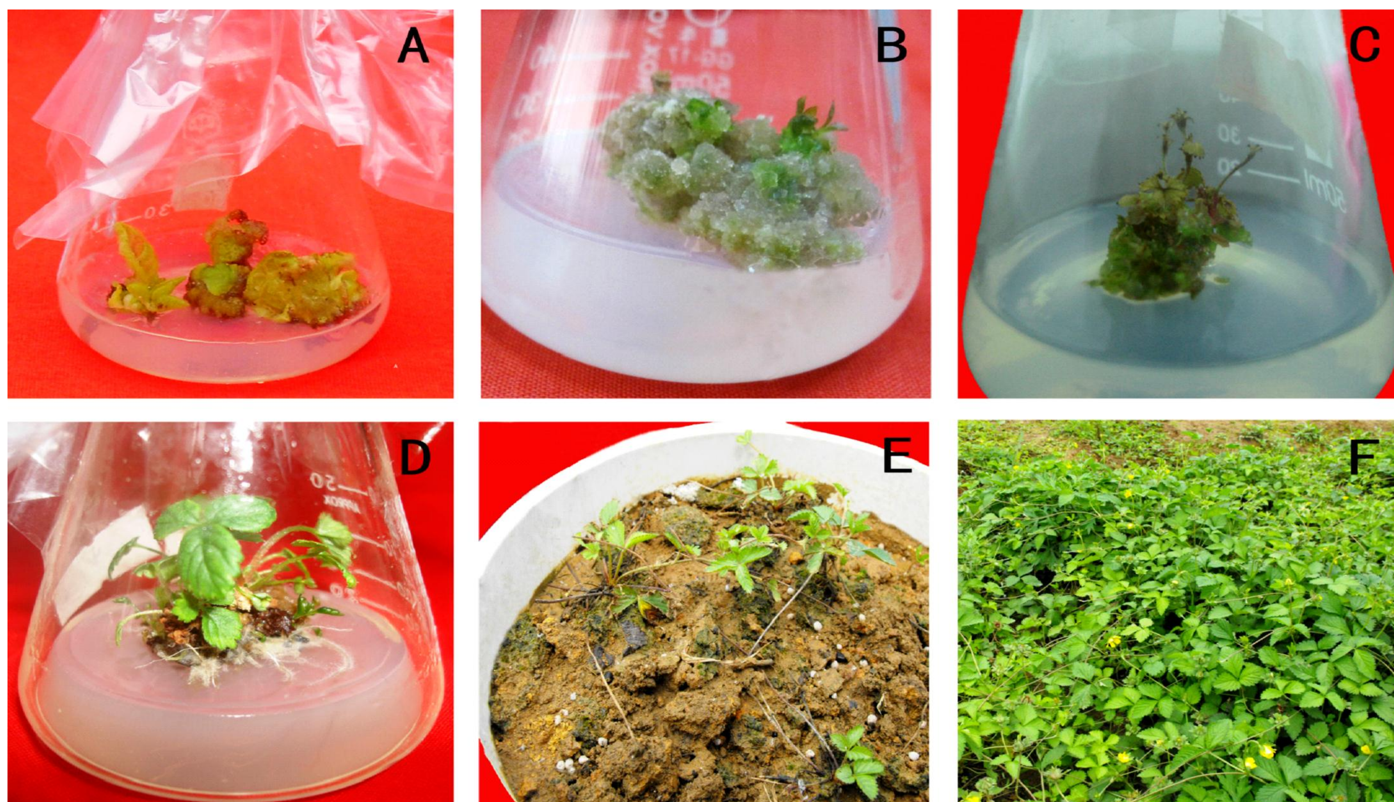

Figure 1. *In vitro* propagation of *Duchesnea indica* (Andr.) Focke. **A**, Induction of callus from explants; callus induction on 6.0 M IAA and 9.0 M BA medium from a hypocotyls; **B**, differentiation of callus by medium with 3.0 M IAA and 2.0 M BA; **C**, shoot organogenesis induced by medium with 2.5 M NAA and 4.5 M BA; **D**, root organogenesis induced by1/2MS medium with 4.0 M IBA and 8.5 M IAA; **E**, individual regenerant acclimatized on a soil mixture with soil: plant ash: compound fertilizer (30:2:1; weight.) in a culture vessel; **F**, acclimated plantlet in the experimental field.

3), and the roots reached an average length of 4.05 cm (Table 3) longer than that of the controls.

Plant performance *ex vitro* was highly dependent on the transplantation substrate as plants transplanted onto the soil mixture with soil: plant ash: compound fertilizer had the best survival rate of 85% (P = 0.0094) and shoot growth (30 to 40 cm) (Figure 1E). The survival of the plants growing in soil only was lower (40%). Plantlets were fully acclimatized at the time of transferring them to pots and no further losses were recorded thereafter. Six months later, all the *in vitro* derived plants were still alive and displayed normal development similar to non-cultured plants.

Conclusion

The micropropagation strategy reported here was characterized by a rapid proliferation of shoots. Such a system could be extremely useful as a model for undertaking studies of a genetic or molecular nature in order to gain insights into the processes governing vegetating in plants, particularly in the Rosaceae. Not only was a high multiplication rate observed but also plantlets easily acclimated to the external environment from tissue culture undergoing normal physiological development (Figure 1F). This is highly advantageous for the conservation of this species and may further aims at the molecular manipulation in *D. indica* (Andr.) Focke.

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