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Effect of perforations of culture vessel cap on growth and leaf microstructure of in vitro plantlets of Artemisia annua L.

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Glandular trichomes on leaf epidermis of Artemisia annua was a major site for artemisinin production. The influence of culture vessel fitted with or replaced with perforated cap was investigated on growth and microstructures of in vitro A. annua leaves. Increment in height and fresh biomass of plantlets regardless of clones (TC1, TC2 and Highland) in culture vessel fitted with normal cap were significantly higher than those cultures with perforated cap after eight weeks of culture. Desiccation of the culture medium was observed in culture vessels fitted with or replaced with perforated caps. Three clones showed different growth profile and sensitivity toward ventilation. T-shaped trichome (non-glandular) and capitates trichome (glandular trichome) were found on adaxial and abaxial epidermis of A. annua leaves. Using perforated cap induced more trichomes on the adaxial surface of leaf for all three tested clones. Stomata of plantlets cultured in culture vessels with normal caps were always opened and opposite trend observed in culture vessels replaced with perforated caps. Results indicated that ventilation due to cap type influenced the growth of in vitro plantlets and microstructures of A. annua leaves.

Key words: Artemisia annua, in vitro plantlets, perforated cap, leaf trichome.

INTRODUCTION

Artemisia annua L. belongs to the genus Artemisia, family Asteraceae, distributed widely in temperate region of central Europe, such as Romania, Hungary, Italy, France and Asia mainly in China and Vietnam (Ziffer et al., 1997). The plant usually single-stemmed reaches two meters in height with alternate branches and deeply dissected, aromatic leaves ranging from 2.5 to 5.0 cm in length. Tiny yellow nodding flowers are displayed in loose panicles containing numerous, greenish or yellowish, bisexual central (disc) florets containing little nectar and pistillate marginal (ray) florets (Ferreira and Janick, 1996). It is an important medicinal plant capable of synthesizing artemisinin, also known as qinghaosu, in the leaves and flowers (Ferreira et al., 1997; Baraldi et al., 2008).

Artemisinin isolated from the aerial parts of A. annua plants have proven to be effective in treating both drug resistant and cerebral malaria parasites (Bilia et al., 2006). Also, artemisinin and its derivatives have promising effect on both agricultural and medicinal insect pests as well as its anticancer, antitumour and antiproliferative activities in human (Posner et al., 1997; Chadwick et al., 2010; Keiser et al., 2010; Tripathi et al., 2000, 2001). Trichomes are specialized tissue that function as leaf temperature or water loss controller, herbivores determent, or produce pest or pollinator-interactive chemicals which stored at the plant surface (Wagner, 1991). Biseriate glandular trichomes were observed in rows along the midrib of adaxial and abaxial leaf epidermis and on the stem of A. annua (Duke and Paul, 1993). These glandular trichomes were found to be the site of sequestration of artemisinin (Duke et al., 1994; Ferreira and Janick, 1996).

Many plant species have been propagated via in vitro
culture techniques. Culture vessels used for in vitro propagation of plants can be considered as a miniature greenhouse or growth chamber (Read, 1990). However, various physiological and pathological problems encountered in in vitro cultures were mainly due to the different physical environment of the in vitro culture vessels as compared to that in a greenhouse (Debergh and Maene, 1984). Different types of vessel closure are used to cover the culture vessels in order to maintain the aseptic condition of the cultures. Types of covers commonly used are cotton plugs (Alizadeh et al., 2010), cellulose stoppers, transparent films, aluminium foils and screw caps made of polypropylene or polyvinylglycine, or combinations of caps with foil or film. Type of vessel cap could result in morphological abnormalities such as hyperhydricity and abnormal growth of the plantlets if the cap is closed. This was mainly due to poor aeration arising from restriction of gaseous exchange between the culture vessel atmosphere and the ex vitro conditions (Blazková et al., 1989; Buddendorf-Joosten and Woltering, 1994). Limited gaseous exchange in the culture vessels has been proven to influence the anatomical and physiological characteristics of some in vitro plants (Majada et al., 1998). In addition, light irradiance, air temperature, carbon dioxide concentration may lead to abnormality in in vitro plantlets (Fujiwara and Kozai, 1995; Zimmerman, 1995). Therefore, to improve the quality and proliferation of plantlets, the growth environment must be maintained at optimum conditions.

Many attempts had been made to develop new types of vessel closure to ensure good gas exchange. To date there are no reports on the effects of perforation of the culture vessel caps which lead to different ventilation system influencing the leaf morphology of A. annua. Therefore, we evaluated the growth of in vitro plantlets of A. annua and the leaf morphology as influenced by the perforations of the vessel caps.

MATERIALS AND METHODS

Plant material

The in vitro plantlets of three different clones of A. annua of Vietnam origin, TC1, TC2 and Highland were established from seeds. They were maintained on Murashige and Skoog (MS) medium (1962) without any plant growth regulators but supplemented with 30 g/l sucrose and 8 g/l of agar (Algas, Chile) and placed under constant temperature of 25 ± 2°C and light intensity of approximately 32.5 μmol m⁻² s⁻¹. The pH of the medium was adjusted to 5.7 before autoclaving (Tomy 325) at 121°C for 11 min under 1.05 kg/cm² pressure. The nodal segments (= 2.0 cm) of the eight-week old in vitro plantlets were used as explants.

Effect of perforated and non perforated culture vessel cap on growth of A. annua plantlets

Three nodal segments of each clone were cultured in each culture vessel containing the same culture medium using non perforated caps with 12 replicates. After four weeks, six of the culture bottle caps were replaced with perforated while the remaining six continued using the non-perforated caps. The experiments were repeated three times. After eight weeks of culture, the height and the fresh biomass of the plantlets were determined.

Effect of replacing with perforated cap on growth of A. annua plantlets

Three nodal segments of each clone were cultured in each culture vessel related to the same culture medium using non perforated caps with 12 replicates. After four weeks, six of the culture bottle caps were replaced with perforated while the remaining six continued using the non-perforated caps. The experiments were repeated three times. After eight weeks of culture, the height and the fresh biomass of the plantlets were determined.

Effect of perforated and non perforated culture vessel caps on trichome morphology of A. annua plantlets

Leaves of eight weeks old A. annua clones were freeze dried using freeze drying machine (CHRIS). The leaf morphology was then viewed using the scanning electron microscope (Leo Supra 50 VP Field Emission). The number, length, width and surface area of glandular and non-glandular trichomes were determined using Soft Image System (SIS) programme.

Effect of replacing with perforated cap on trichome morphology of A. annua plantlets

Leaves of three different clone cultured in culture vessels replaced with perforated caps and the remaining with normal caps were fixed in McDowell-Trump fixative prepared in 0.1 M phosphate buffer or cacodylate buffer (pH 7.2) at 4°C overnight. The leaves were then washed with 0.1 M phosphate buffer for 30 min and postfixed in 1% Osmium tetroxide (OsO₄) solution at 25°C for 1 to 2 h. The specimens were then rinsed with distilled water for 20 minutes and continued with dehydration process using 50% ethanol, 75% ethanol, 95% ethanol and 100% ethanol. The dehydrated tissues were immersed in hexadecylsilazane (HMDS) for 10 min. HMDS from the specimen vial was decanted and the specimen vial with the tissue was left in the desiccator to air dry at room temperature. The specimens were then mounted by double adhesive tape on aluminium stubs, and coated with thin gold layer (40 to 60 mm) using Bio-Rad SEM coating system. Leaf morphology was examined with the aid of Scanning Electron Microscope (SEM) at 8 to 10 kV. The number of glandular and non-glandular trichomes were determined and the morphology of stomata were observed using Soft Image System (SIS) programme.

Experimental design and statistical analysis

The experiment was carried out using independent two sample case for each clone. The data (increase in height and biomass) were analysed using Student t-test at p ≤ 0.05. Statistical analyses were performed using SPSS 16.0 software for windows.
RESULTS AND DISCUSSION

Different *A. annua* clones showed different growth kinetics but all the clones cultured in culture vessel with normal cap (non-perforated) showed significantly faster growth compared to plantlets grew in culture bottles with perforated cap (Figure 2). After eight weeks of culture, the plantlets of all the three clones attained approximately the same height (8 - 8.5 cm) when they were cultured in the culture vessels using the normal cap. Same was observed for those culture using the perforated caps except their height was very much shorter (4 - 4.5 cm). The fresh biomass of the plantlets was also found to be higher for the different clones that were cultured in the culture bottles with the normal caps (Figure 3). However, the plantlets of Highland clone (1.8 g) were of lower biomass as compared to TC1 (2.5 g) and TC2 (2.1 g) when cultured in culture vessels with normal caps. The fresh biomass of the different clones was not significantly different in vessels with perforated cap (Figure 3). The increment in plant height for continuously cultured in normal cap culture vessels were not significantly different for all *A. annua* clones as compared to the situation where the caps were replaced with perforated caps in between. The growth of the plantlets were not influenced by the replacement of normal cap to perforated cap on the forth weeks of culture. This was because the plantlets were entering into the exponential growth phase at that time. Rapid cell division during this phase might enable the plantlets to be more resistance to change of the *in vitro* environment condition. The non-significant difference in height was also due to the high variation of the *A. annua*
Figure 2. Growth kinetics of *A. annua* plantlets (A: TC1; B: TC2; C: Highland) in culture vessels with normal caps and perforated caps. Mean values at week eight followed by the same alphabet were not significantly different (T-test, $p \leq 0.05$). Bars represent mean ± standard error.

Plantlets especially the Highland clone. This was because Highland clone is a wild type whereas clone TC1 and TC2 are the selected clones. The fresh biomass also showed no significant different for TC2 and Highlands clones, but there was significant difference for TC1 clones when the culture vessels were replaced with perforated caps (Figure 6). There were two types of trichomes found on *A. annua* leaves, T-shaped trichome...
Figure 3. Biomass of *A. annua* clones after eight weeks of culture in culture vessel with normal cap and perforated cap. Mean values for each clone followed by the different alphabet were significantly different (T-test, \( p \leq 0.05 \)). Bars represent mean ± standard error.

*Artemisia annua* clone

<table>
<thead>
<tr>
<th></th>
<th>Normal cap</th>
<th>Perforated cap</th>
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<tbody>
<tr>
<td>TC1</td>
<td></td>
<td></td>
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<tr>
<td>TC2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Highland</td>
<td></td>
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Figure 4. Influence of culture vessel caps (a: normal cap; b: perforated cap) on the medium of *A. annua* after seven weeks of culture.

(non-glandular) and capitates trichome (glandular trichome) (Figure 7). The distribution of trichomes found on the leaf of *A. annua* were found to be affected by perforated cap. The use and replacement of perforated cap induced the formation of higher number of trichomes (glandular and non-glandular) for all the three different
clones at the adaxial of leaf compared to non-perforated cap. More non-glandular trichomes were found at the abaxial of the leaf when using the perforated cap. However, the number of glandular trichomes at the abaxial of the leaf for TC1 and TC2 clones were not affected by the type of caping but was significantly different for the Highland clone (Table 1).

The result indicated that the environment inside the culture vessel fitted with perforated cap enhanced the development of trichomes in *A. annua* as compared to normal cap. It was reported that low humidity enhanced development of trichomes in *Wigandia urens* (Pérez-Estrada et al., 2000). Perforated cap culture vessel created lower humidity for the *in vitro* microenvironment which enhanced the development of trichomes compared to non-perforated cap culture vessel. Number and type of trichomes regardless of the type of cap used were found to be more distributed on the adaxial parts. However,
Figure 6. (a) Height increased and (b) fresh biomass of *A. annua* clones after four weeks of replacement with perforated cap. Mean values for each clone followed by the same alphabet were not significantly different (T-test, $p \leq 0.05$). Bars represent mean ± standard error.

non-glandular trichome of TC1 clones cultured in normal cap and glandular trichome of TC2 clone cultured in normal cap were found most on abaxial part. Non-glandular trichomes of Highland clones were not found on adaxial and abaxial surface (Table 2).

The stomata found on the leaf of plantlets grew in non-perforated cap were open (Figure 8a) due to high humidity of the microenvironment. For plantlets cultured in culture vessels with perforated cap, the leaf stomata were closed (Figure 8b) due to lower humidity and higher
Figure 7. Two types of trichomes observed under scanning electron microscope (SEM): a: T-shaped trichome (Non-glandular); b: capitates trichome (glandular trichome).

Table 1. Effect of perforated and non perforated culture vessel cap on the number of trichomes of A. annua leaf on both leaf adaxial and abaxial surface (300 x 300 µm area).

<table>
<thead>
<tr>
<th>A. annua clones</th>
<th>Normal cap</th>
<th>Perforated cap</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>Adaxial</td>
<td>Abaxial</td>
</tr>
<tr>
<td></td>
<td>G  N</td>
<td>G  N</td>
</tr>
<tr>
<td>TC1</td>
<td>1a 1a</td>
<td>3a 1a</td>
</tr>
<tr>
<td>TC2</td>
<td>1a 1a</td>
<td>1a 1a</td>
</tr>
<tr>
<td>Highland</td>
<td>2a 1a</td>
<td>1a 2a</td>
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G: Glandular trichome; N: Non-glandular trichome. Mean values for each clone followed by the same alphabet are not significantly different (T-test, p ≤ 0.05).

Table 2. Effect of replacing with perforated cap on the number of trichomes per mm2 of A. annua leaf on both leaf adaxial and abaxial.

<table>
<thead>
<tr>
<th>A. annua clones</th>
<th>Normal cap</th>
<th>Perforated cap</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>Adaxial</td>
<td>Abaxial</td>
</tr>
<tr>
<td></td>
<td>G  N</td>
<td>G  N</td>
</tr>
<tr>
<td>TC1</td>
<td>43 ± 9</td>
<td>44 ± 18</td>
</tr>
<tr>
<td>TC2</td>
<td>19 ± 2</td>
<td>13 ± 1</td>
</tr>
<tr>
<td>Highland</td>
<td>60 ± 8</td>
<td>6 ± 3</td>
</tr>
</tbody>
</table>

G: Glandular trichome; N: Non-glandular trichome.

ventilation. Therefore, regulation of stomata aperture was needed for the plantlets to prevent water lost. Plantlets in non-perforated cap culture vessel would prevent greater water lost as compared to plantlets in culture vessel with perforated cap (Deccetti et al., 2008). However, in in vitro rose culture, the leaf stomata were found to be always open if the ventilation was high (Sallanon and Maziere, 1992).

In conclusion, the effect of ventilation on the growth and leaf trichomes of A. annua plantlets depending on the clones were: Wild type clone (Highland) showed high variation compared to the selected clones (TC1 and TC2).
Culture vessel with normal cap increased the biomass and number of leaves of *A. annua* plantlets regardless of the clone type. Whereas, culture vessel with perforated cap increased the number of glandular and non-glandular trichomes on both adaxial and abaxial part of leaf.

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**REFERENCES**


