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Full Length Research Paper

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, Itali, 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 lose 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).

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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 phsiological 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 approximely 32.5 µmol m^{-2} s^{-1}. The pH of the medium was adjusted to 5.7 before autoclaving (Tommy 325) at 121°C for 11 min under 1.05 kg/cm^2 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 growh of *A. annua* plantlets

The nodal segments (≈ 2.0 cm) of *A. annua* (TC1, TC2, Highland clones) were cultured into glass culture vessels (250 ml) containing 40 ml MS medium without any plant growth regulators and supplemented with 30 g/l sucrose and 8 g/l of agar (Algas, Chile). Three explants were used for each culture vessel and six experimental units were used to test each type of capping system for each clone. The clear screwed caps of the culture vessels were either non-perforated or perforated with seven perforations of 0.5 cm in diameter. The undersides of perforated caps were layered with two pieces of white tracing paper (Figure 1). The height of each plantlets for each clone was determined every week until eight weeks of culture. The eight weeks old plantlets grown in the culture vessels with normal cap and perforated cap were removed to determine the biomass.

Effect of replacing with perforated 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 bottles caps were replaced with perforated caps 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 cacoadylate 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 (OsO_4) 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 hexathyldisilazane (HMDS) for 10 min. HMDS from the specimen vial was decanted and the specimen vial with the tissue was left in the dessicator 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 $p \leq 0.05$. Statistical analyses were performed using SPSS 16.0 software for windows.

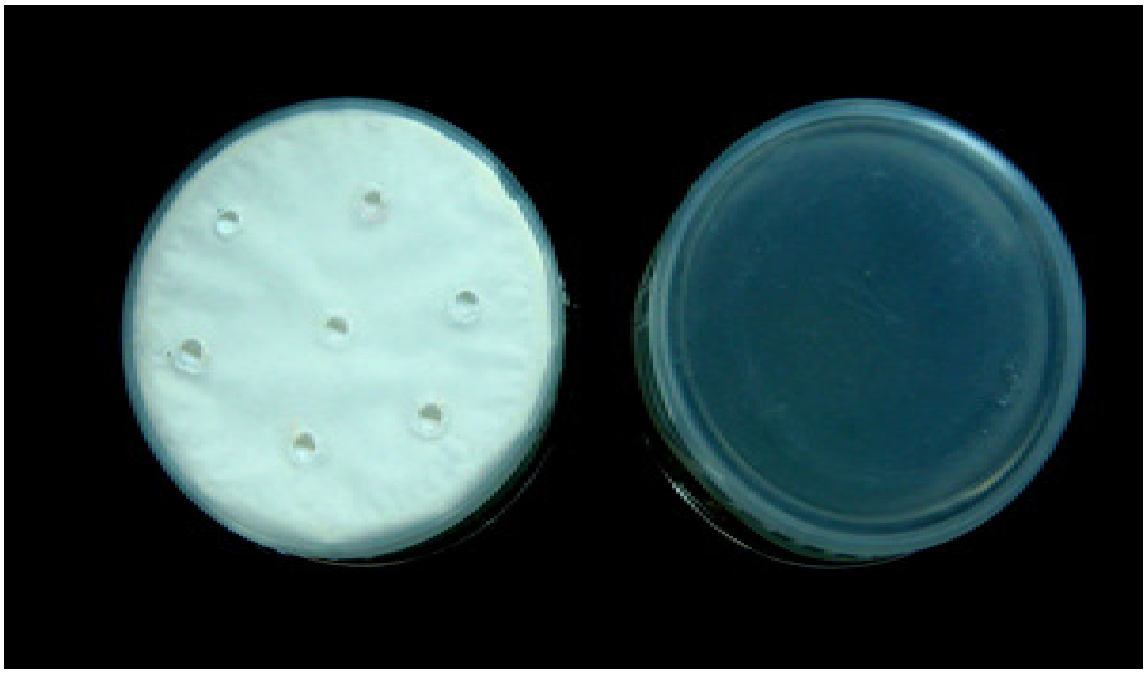

Figure 1. Perforated cap layered with white tracing paper (left) and normal clear screw cap (right).

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. This results thus indicated that higher humidity that was created by the culture vessels with normal caps induced better growth for the *in vitro* plantlets of *A. annua*. The relative humidity in non-ventilated culture vessel was higher compared to perforated cap culture vessels. This facilitated the normal growth of the plantlets (Fujiwara and Kozai, 1995). However, the study of Mills et al. (2004) showed contradicting result whereby no ventilation produced less elongated shoots and low dry weight biomass compared to those cultured using intermediate ventilation.

The culture medium dessication was observed in perforated cap culture vessel (Figure 4). This phenomena was in agreement with other reports that desiccation of the culture medium was observed when there was increased ventilations (McCown and Sellmer 1986; Sallanon and Maziere, 1992). Gas exchange in culture vessel was found to be higher (Kozai et al., 1986) when plastic cap was used as culture vessel closure instead of metal caps. A possible explanation of this phenomena was that gas exchange occured through the fitting between the culture vessel and the cap (Fal et al., 2002). Therefore, plastic cap with perforations used in our study increased the gaseous exchange and desiccation and thus resulted in yellowish browning and wrinkled of the leaves and less root formation for all plantlets of the three different clones. However, those grown in culture vessel with normal cap produced green healthy plantlets with expanded leaves and more root formation (Figure 5). 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 conditon. The non-significant difference in height was also due to the high variation of the *A. annua*

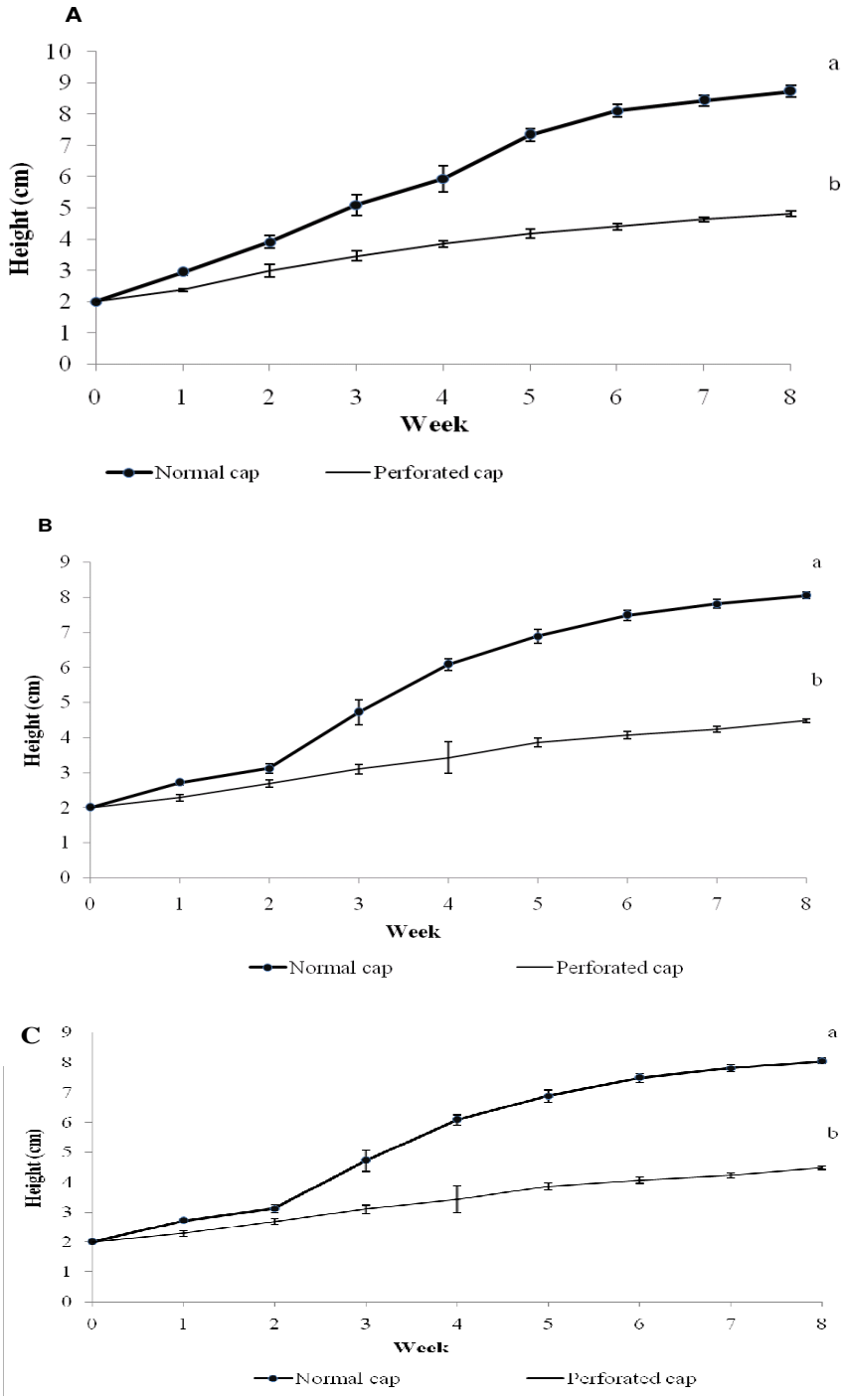

Figure 2. Growth kinetics of *A. annua* plantlets (A: TC1; B: TC2; C: Highland) in culture vessels with normal caps and perforated caps. Mean values at week eight followed by the same alphabet were not significantly different (T-test, $p \leq 0.05$). Bars represent mean ± standard error.

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

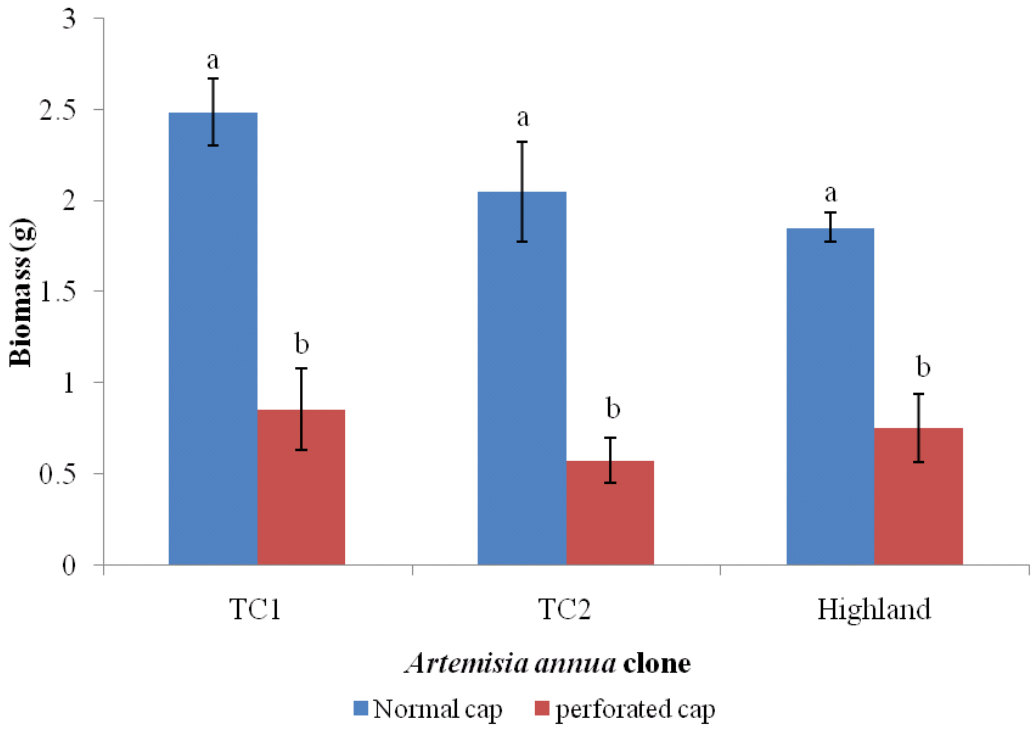

Figure 3. Biomass of *A. annua* clones after eight weeks of culture in culture vessel with normal cap and perforated cap. Mean values for each clone followed by the different alphabet were significantly different (T-test, $p \leq 0.05$). Bars represent mean ± standard error.

a **b**

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

Figure 5. Plantlets of *A. annua* in culture vessel with normal cap (a) TC1; (b) TC2; (c) Highland and perforated cap (d) TC1; (e) TC2; (f) Highland.

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 peroforated 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,

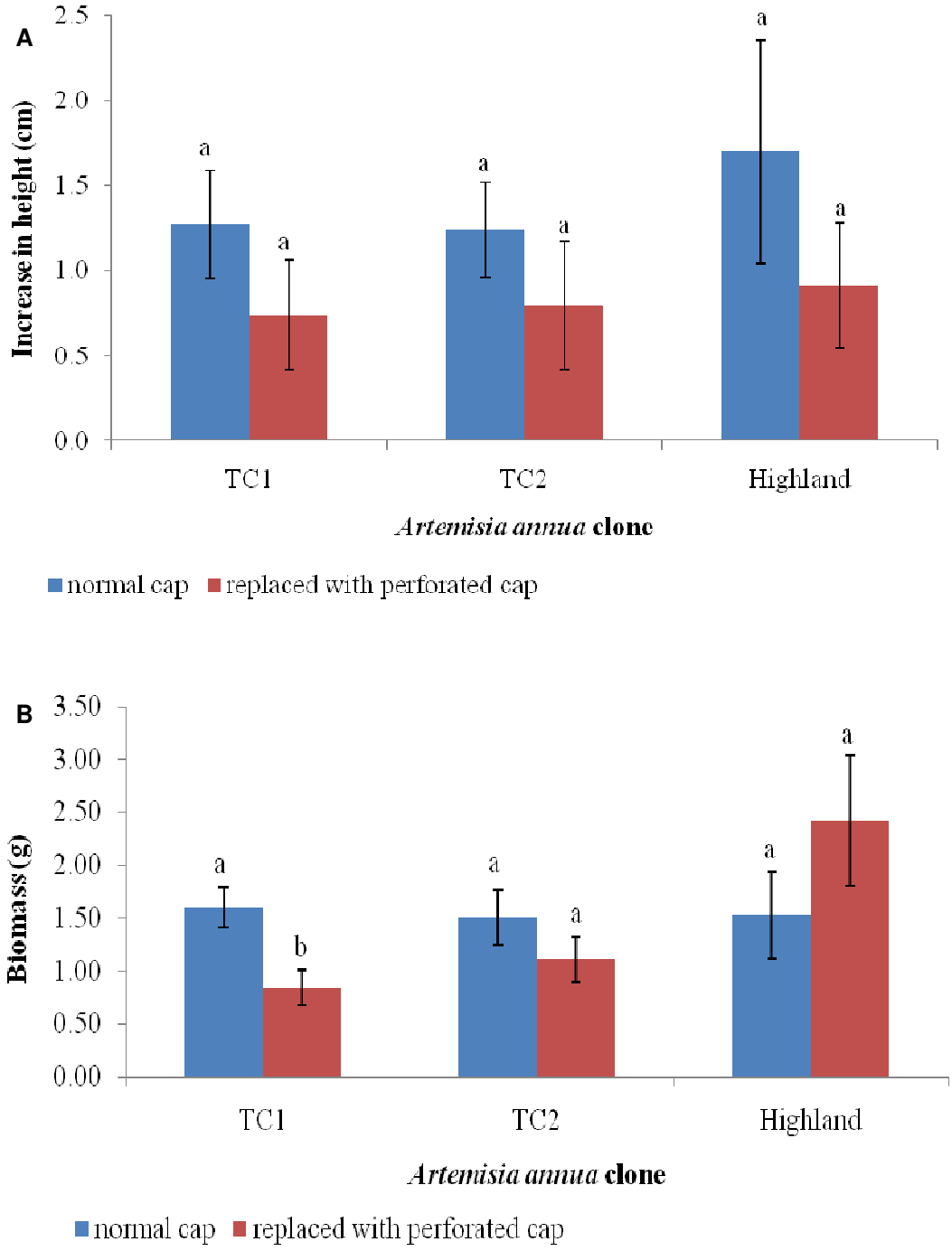

Figure 6. (a) Height increased and (b) fresh biomass of *A. annua* clones after four weeks of replacement with perforated cap. Mean values for each clone followed by the same alphabet were not significantly different (T-test, $p \leq 0.05$). Bars represent mean ± standard error.

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

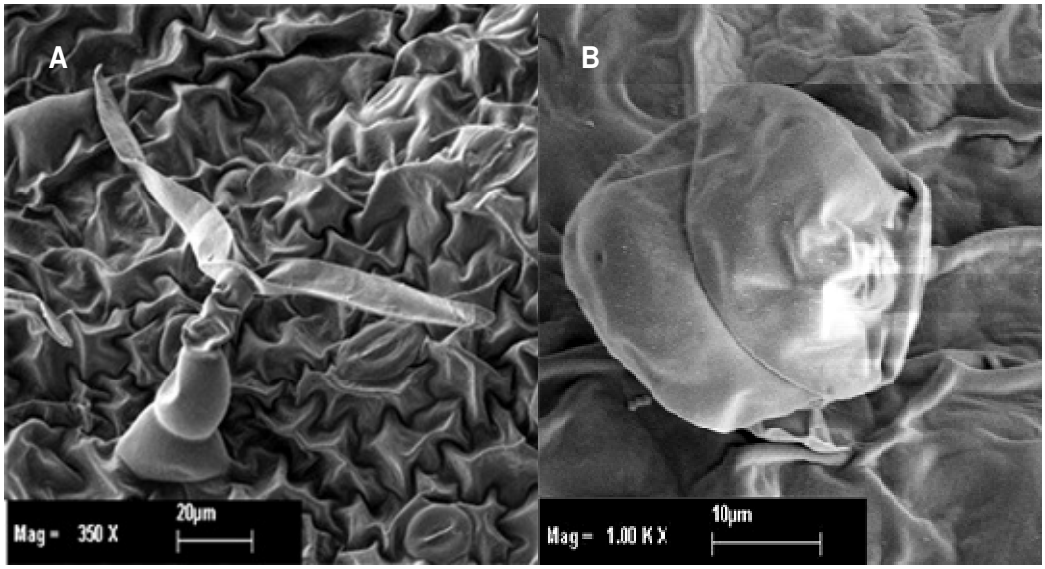

Figure 7. Two types of trichomes observed under scanning electron microscope (SEM): a: T-shaped trichome (Non-glandular); b: capitates trichome (glandular trichome).

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).

A. annua clones	Normal cap				Perforated cap			
	Adaxial		Abaxial		Adaxial		Abaxial	
	G	N	G	N	G	N	G	N
TC1	1a	1a	3a	1a	4b	3b	3a	3b
TC2	1a	1a	1a	1a	4b	4b	1a	4b
Highland	2a	1a	1a	2a	5b	4b	6b	6b

G: Glandular trichome; N: Non-glandular trichome. Mean values for each clone followed by the same alphabet are not significantly different (T-test, $p \leq 0.05$).

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.

A. annua clones	Normal cap				Perforated cap			
	Adaxial		Abaxial		Adaxial		Abaxial	
	G	N	G	N	G	N	G	N
TC1	43 ± 9	44 ± 18	12 ± 4	39 ± 18	27 ± 6	30 ± 7	28 ± 9	19 ± 7
TC2	19 ± 2	13 ± 1	30 ± 7	9 ± 2	33 ± 1	24 ± 3	7 ± 1	8 ± 0
Highland	60 ± 8	6 ± 3	12 ± 1	6 ± 6	96 ± 6	6 ± 2	78 ± 9	26 ± 10

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).

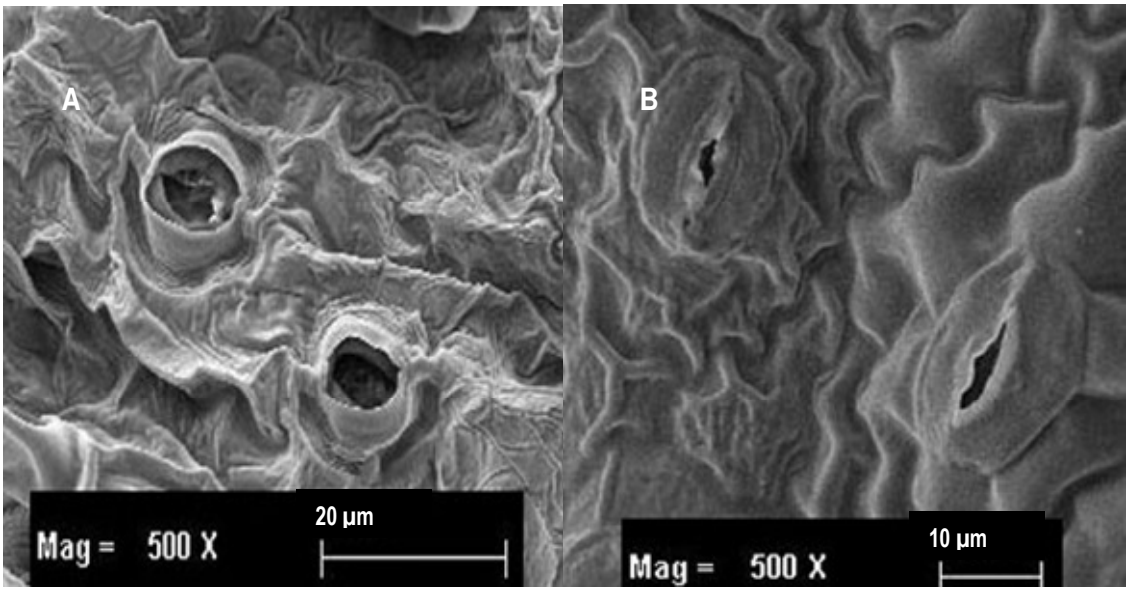

Figure 8. Stomata opening on adaxial leaf epidermis of *A. annua* plantlets cultured in culture vessels fitted with (a) normal and (b) perforated cap .

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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