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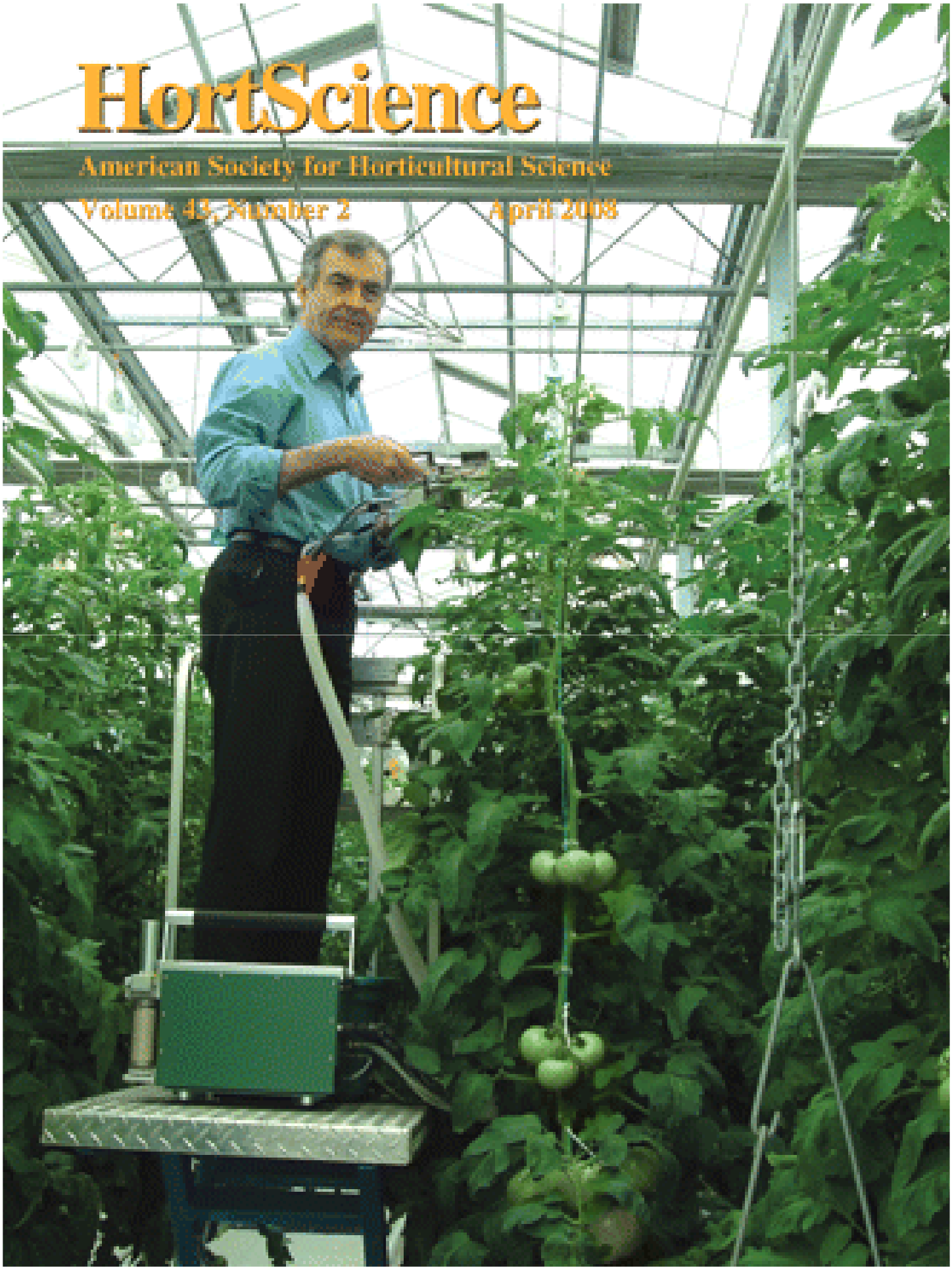

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In Vitro Clonal Propagation of Vanilla (*Vanilla planifolia* 'Andrews')

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Abstract. A complete and efficient regeneration protocol was developed for *Vanilla planifolia* 'Andrews', an endangered orchid species that represents an important crop in several tropical countries. Axillary buds excised from the first to the eighth node, considering the first to fourth nodes as "young" (zone 1) and the fifth to eighth as "mature" (zone 2), were cultured on Murashige and Skoog (MS) medium supplemented with 5.73, 7.64, 9.55, or 11.46 μM 6-benzylaminopurine (BA) for shoot induction and in combination with 4.45 μM naphthalene acetic acid (NAA) to induce multiple shoot proliferation. Cytokinin concentration and bud position in the stem had a significant effect on the number of shoots regenerated. The greatest shoot formation per explant, for the two tested zones, was obtained with 9.55 μM BA on MS medium supplemented with 100 mg·L^{-1} myoinositol, 150 mg·L^{-1} citric acid, 100 mg·L^{-1} ascorbic acid, and 20 g·L^{-1} sucrose. Young buds from zone 1 were able to form an average of 18.5 ± 2.4 shoots per explant, whereas buds from zone 2 induced a maximum of 11.0 ± 1.0 shoots per explant. Plants of 2 to 3 cm height developed a root system in half-strength MS medium supplemented with 0.44 μM NAA and, once they reached 5 cm height on average, were transferred to greenhouse conditions for their acclimatization where a 100% rate survival was achieved. The optimal use of both young and mature buds from each mother plant to induce adventitious shoots permitted a marked increase in the number of shoots per explant. By using all buds from the upper stem part (zone 1 + zone 2) and subculturing every 90 d, the multiplication rate was 1.1 to 1.86 × 10^{6} shoots per bud per year.

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Vanilla planifolia 'Andrews' (vanilla) is the natural source of the flavoring and aromatizing vanillin, the main flavor component of cured vanilla beans used in many industries (food, liquor, soft drinks, pharmaceutical, cosmetic, tobacco, and handcrafts) and considered to be of better quality than the synthetic product. *V. planifolia* 'Andrews', also known as *Vanilla fragrans* (Salisb) (Bouriquet, 1954), is a tropical climbing orchid with origins in Mexico and Central America (Funk and Brodelius, 1990). Despite Mexico being considered as the origin and domestication center of *Vanilla planifolia* 'Andrews', now this is an endangered Mexican species, mainly as a result of reduced multiplication rates of this species through traditional production techniques, unsuitable farming labor, and several fungal damages and infections. Because of this situation, Mexican vanilla growers confront many challenges when producing vanilla vines that meet official standards, foreign competition, and miss the chance to produce enough plant material to satisfy an increasing demand of vanilla vines. Therefore, more efficient methods are required for large-scale elite plants production. Morel (1960) was the first to demonstrate the possibility of cloning virus-free orchids through apex culture (Goh, 1983). However, in monopodial orchids such as vanilla, meristem culture requires the removal of the apical shoot, which results in retarded growth and development of mother plants. Over the last few decades, the possibility of propagating orchids in vitro using other parts of the plant as explants has been investigated. There are reports of in vitro regeneration for this species through callus culture (Davidonis and Knorr, 1991; Gu et al., 1978), protocorms and root apex (Philip and Nainar, 1986), axillary buds (George and Ravishankar, 1997; Giridhar and Ravishankar, 2004; Kononowicz and Janick, 1984), and shoot tips (Geetha and Sudheer, 2000). However, the efficiency of these reported protocols is still low, probably as a result of phenolic compounds that accumulate in explant tissues during in vitro establishment. Given the increased interest and worldwide demand for elite *Vanilla planifolia* A. plants, we established an efficient large-scale micropropagation protocol for this species through the optimal use of axillary buds from each mother plant to achieve the highest number of adventitious shoots per bud per plant and to promote successful acclimatization of the regenerated plants.

Materials and Methods

Explant preparation. Nodes were collected from 3-years-old vanilla (*V. planifolia* A.) plants grown in Papantla, Veracruz, México. Axillary buds, between 3 and 5 mm in diameter, were excised from the first to the eighth node of the stem and eventually distinguished in two zones: zone 1, including buds between first to fourth nodes (young buds), and zone 2, including buds between fifth and eighth nodes (mature buds).

Isolated buds were disinfected with a surfactant solution (1 to 2 drops of Tween-20; ICI Americas; in 1 L distilled water) and washed under a slow flow of tap water for 45 min, then a higher flow for 10 more min. Subsequently, buds were consecutively submerged in commercial sodium hypochlorite (6% active chlorine) diluted at different

the micropropagation process more efficient by developing a system with a highest shoot proliferation (18.5 ± 2.4 shoots) including shorter time periods for induction (30 to 45 d) and multiple shoot proliferation (30 d) is proposed. We determined the use of 9.55 μM BA, a higher concentration than used in other reported protocols, for both induction and proliferation steps; however, it simplifies and enhances the efficiency of our developed system by reducing the incubation time required for shoot induction and proliferation. George and Ravishankar (1997) reported a protocol that included 7.64 μM BA to induce an average of 5.7 shoots per explant in a 90-d period, which means a longer time for shoot induction. In general, the protocols previously reported require 4 to 6 months to complete only shoot induction and proliferation stages. Ganesh et al. (1996) mentioned that the main difficulties in the propagation of vanilla species through node culture were: 1) the adverse effect of ethylene that can be accumulated in vessel cultures and inhibit shoot proliferation; and 2) highly condensed internodes. To overcome these problems, they supplemented all used media with 20 mg·L^{-1} $AgNO_3$ as an ethylene inhibitor and 0.1 mg·L^{-1} GA_3 to induce shoot elongation; additionally, they used 1 mg·L^{-1} BA to induce shoot proliferation, and the subculture time was extended to 135 d. In our study, subculturing into media lacking $AgNO_3$ and GA_3, and supplemented with BA (9.55 μM) at 30-d subculture periods, allowed efficient shoot proliferation with normal elongated internodes, probably as a result of shorter subculturing times and a higher BA concentration, which avoid ethylene accumulation, and shoots 5 cm in length could be obtained 3 months after culture initiation. It is widely known that *V. planifolia* 'Andrews' is the only orchid species of great industrial value, because it is the natural source of the flavoring compound known as vanilla; therefore, a highly reliable clonal propagation system is required to produce uniform plants with appropriate characteristics for intensive cultivation. As a result of this study, a rapid, simple, and efficient large-scale protocol for in vitro clonal propagation of *Vanilla planifolia* 'Andrews' was established, which is probably the most efficient procedure reported to date. Establishing rapid and simple plant production procedures with higher multiplication rates than those obtained with traditional methods is undoubtedly of great value. With this efficient micropropagation protocol, we are contributing to improve the large-scale production of elite *Vanilla planifolia* 'Andrews' plants widely used for commercial production and industrial purposes.

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