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SMOKE-SATURATED WATER FROM FIVE GRASSES GROWING IN JAPAN INHIBITS *IN VITRO* PROTOCORM-LIKE BODY FORMATION IN HYBRID *CYMBIDIUM*

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Abstract: Smoke derived from the burning of plant material has been shown to stimulate seed growth of several species. In addition, several studies have reported that when smoke is condensed with water, smoke-saturated water (SSW) can also stimulate the germination of orchid seeds. In this study, SSW was derived from burning the aerial part of five grasses growing in the wild in Shikoku, Japan (*Arundinella hirta* (Thunb.) C. Tanaka var. *hirta*, *Microstegium japonicum* (Miquel) Koidzumi, *Miscanthus sinensis* Andersson, *Paspalum thunbergii* Kunth ex Steud., *Themeda triandra* Forssk. var. *japonica* (Willd.) Makino), all of which flower between August and October. SSW was added at three concentrations (1, 5, 10%, v/w) to solid, agarized Teixeira *Cymbidium* (TC) medium to assess the impact on *in vitro* organogenesis of hybrid *Cymbidium*, specifically on new protocorm-like body (*neo*-PLB) formation. The SSW of all five species strongly inhibited the formation of *neo*-PLBs at all concentrations relative to the control (no SSW added). Since PLBs are considered to be the equivalent of somatic embryos in orchids, and since SSW is able to stimulate the germination of zygotic embryos in other plant families, the mechanism of action is clearly different between zygotic and somatic embryos.

Key words: karrakinins, orchid, PLB, smoke-saturated water, Teixeira *Cymbidium* (TC) medium.

Introduction

DE LANGE & BOUCHER (1990) were the first to note, in the South African Cape fynbos, how fynbos plant-derived smoke or its aqueous extract, i.e., smoke-saturated water (SSW), could stimulate seed germination of *Audouinia capitata* (L.) Brongn., a threatened monotypic fynbos species. Burning fynbos (aerial parts) could also stimulate seed germination of fire-climax grass *Themeda triandra* Forssk. (syn. *Triandra australis* (R.Br.) Stapf) [BAXTER & al. 1994]. Since these studies, several other studies have emerged showing how burning plant material, particularly in South Africa and in Australia, has led to seed effective germination of many species. This may be an ecological adaptation since these dry, Mediterranean-type climates receive little rainfall and are often prone to bush or wild fires, therefore the ability to use elements of both fire and water would enhance survival. BROWN & al. (2003) noted, however, that the seeds of not all fynbos species are stimulated to germinate in response to burning fynbos smoke: seed germination of members of the Asteraceae, Bruniaceae, Crassulaceae, Ericaceae, Geraniaceae, Mesembryanthemaceae, Proteaceae and Restionaceae was stimulated, but not of members of the Amaryllidaceae, Hyacinthaceae or Iridaceae. Similarly, ROCHE & al. (1994) could only germinate seed of 45 out of 94 species of native Western Australian plants that are

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normally hard to germinate by exposing dormant seed to cold smoke derived from burnt native vegetation. Dormant seeds of *Emmenanthe penduliflora* Benth., an annual from the California chaparral, were induced to germinate by smoke or vapors emitted from smoke-treated sand or paper [KEELEY & FOTHERINGHAM, 1997]. Seedlings of two South African indigenous medicinal plants, *Albuca pachychlamys* Baker and *Tulbaghia violacea* Harv., germinated with smoke solutions, showed improved plant growth characteristics *in vitro*, but a third plant, showed no improvement relative to non-smoke treatments [SPARG & al. 2005]. Only one (*Aristolochia debilis* Sieb. & Zucc.) out of 13 species growing in China showed improved germination in the presence of a commercial Australian-derived SSW product [ZHOU & al. 2013].

The germination-promoting compounds present in plant- and cellulose-derived smoke include a butenolide, 3-methyl-2*H*-furo [2,3-*c*] pyran-2-one [FLEMATTI & al. 2004], a non-toxic compound [VERSCHAEVE & al. 2006], renamed karrikinolide 1 (KAR_1) [LONG & al. 2010], or alkyl-substituted 2H-furo[2,3-c]pyran-2-ones [FLEMATTI & al. 2009]. KAR_1 improved somatic embryogenesis of an important, bamboo-like commercial horticultural species, *Baloskion tetraphyllum* (Labill.) L.A.S. Johnson & B.G. Briggs APNI [MA & al. 2006] and SSW enhanced secondary somatic embryogenesis in *Brassica napus* L. [ABDOLLAHI & al. 2012]. Other KARs have since been identified (KAR_2, KAR_3, KAR_4; NELSON & al. 2009).

Since KARs are relatively non-volatile in nature (m.p. 118–119 °C) and soluble in water [FLEMATTI & al. 2004], they can be concentrated in the aerosol component of smoke created from burning fresh plant material and transported by steam distillation. Using this method to create SSW, SSW was shown to enhance seed germination *in vitro* of several orchid species: *Vanda parviflora* Lindl. [MALABADI & al. 2008], *Xenikophyton smeeanum* (Rchb.f.) Garay [MALABADI & al. 2011], *Pholidota pallida* Lindl. [MULGUND & al. 2012], and *Oberonia ensiformis* (Rees) Lindl. [MALABADI & al. 2012]. It was also the method used in this study.

There is no English literature pertaining to the use of Japanese vegetation-derived SSW for improving seed germination. Based on this gap in the literature, and based on the assumption that a protocorm-like body (PLB) is a somatic embryo in orchids [TEIXEIRA DA SILVA & TANAKA, 2006], this study was conducted to assess the impact of SSW derived from five grasses growing wild in Shikoku, Western Japan, on the induction of new or *neo*-PLBs and *in vitro* organogenesis of hybrid *Cymbidium*. No such study exists for *Cymbidium* Swartz [HOSSAIN & al. 2013; TEIXEIRA DA SILVA, 2013a]. Even though many media can support the induction and development of *Cymbidium* PLBs *in vitro* [TEIXEIRA DA SILVA & al. 2005], Teixeira *Cymbidium* (TC) No. 1 medium [TEIXEIRA DA SILVA, 2012] was used in this study.

Materials and methods

All protocols (experimental design, chemicals, reagents, explant preparation, treatment analysis and advice) strictly follow TEIXEIRA DA SILVA & al. (2005, 2006a, 2006b), TEIXEIRA DA SILVA (2013b), and TEIXEIRA DA SILVA & DOBRÁNSZKI (2013). All chemicals and reagents, including plant growth regulators (PGRs), were of the highest analytical grade available and were purchased from either Sigma-Aldrich (St. Louis, USA), Wako Chemical Co. (Osaka, Japan) or Nacalai Tesque (Kyoto, Japan), the cheapest choice at the highest tissue-culture grade, unless specified otherwise.

Plant material and culture conditions

PLBs of hybrid *Cymbidium* Twilight Moon 'Day Light' (Bio-U, Tokushima, Japan) originally developed from shoot-tip culture on VACIN & WENT (VW, 1949) agar medium without PGRs, were induced and subcultured (PLB induction and proliferation medium) every two months on Teixeira *Cymbidium* (TC) No. 1 medium [TEIXEIRA DA SILVA, 2012], which contains 0.1 mg/l α-naphthaleneacetic acid (NAA) and 0.1 mg/l kinetin (Kin), 2 g/l tryptone and 20 g/l sucrose, and solidified with 8 g/l Bacto agar (Difco Labs., USA). All media were adjusted to pH 5.3 with 1 N NaOH or HCl prior to autoclaving at 100 KPa for 17 min. Cultures were kept on 40 ml medium in 100-ml Erlenmeyer flasks, double-capped with aluminium foil, at 25 °C, under a 16-h photoperiod with a light intensity of 45 μmol/m^2/s provided by 40-W plant growth fluorescent lamps (Homo Lux, Matsushita Electric Industrial Co., Japan). Longitudinally dissected as two pieces of PLB (3-4 mm in diameter) segments [TEIXEIRA DA SILVA, 2013b], 10/flask, were used as explants for *neo*-PLB induction and proliferation.

Response of Cymbidium to smoke-saturated water

The effect of SSW from five grass (Poaceae) species growing in Japan (*Arundinella hirta* (Thunb.) C. Tanaka var. *hirta*, *Microstegium japonicum* (Miquel) Koidzumi, *Miscanthus sinensis* Andersson, *Paspalum thunbergii* Kunth ex Steud., *Themeda triandra* Forssk. var. *japonica* (Willd.) Makino), all of which flower between August and October, on *neo*-PLB formation from half-PLBs was assessed by adding 0 (control), 1%, 5% and 10% (v/w) SSW of each grass to solid TC medium (without PGRs) using the experimental design of TEIXEIRA DA SILVA (2012). All five species, collected from Kagawa, Shikoku, were identified by comparison with internet sites and Japanese field guides such as OHWI (1984). No specimens were deposited in a herbarium. The logic behind removing PGRs was to assess whether SSW could effectively induce *neo*-PLBs in the absence of PGRs and thus test their PGR-like ability. Plant material was collected between August and October when plants were in full flowering. Several hundred flowering stems, weighing a few kilograms each, were harvested from the base, just above the soil line, to allow for future restoration and resprouting. Aerial parts were bundled and placed in cooled conditions and transported to a shaded area with a net screen. Using a Clevenger-type apparatus (self-designed) in Fig. 1, material was set alight, 100 ml of tap water was evenly sprinkled over the plant material (100 ml/kg), each species separately, and the SSW that condensed was collected into separate 500-ml Erlenmeyer flasks. Flasks were placed on ice, covered with a single layer of aluminium foil, transported to the laboratory, kept at 4 °C and used within 24 h. A single stock for each grass species was used for all dilutions and for all replications. Prior to adding to TC medium at the desired concentration, each SSW was passed through a 22 μm Millipore filter, and pH was adjusted to 5.8 prior to adding Difco agar and autoclaving. Explants were photographed using a stereo light microscope and/or a digital camera. Chemical analysis of the SSW was not conducted.

Statistical analyses

Experiments were organized in a randomized complete block design (RCBD) with three blocks of 10 replicates per treatment (i.e., SSW concentration). All experiments were repeated in triplicate (n = 30, total sample size per treatment). The resulting organogenic outcome (*neo*-PLB or root response) was scored visually after 60 days. Data was subjected to analysis of variance (ANOVA) with mean separation by Duncan's multiple range test (DMRT) using SAS® version 6.12 (SAS Institute, Cary, NC, USA). Significant differences between means were assumed at $P \leq 0.05$.

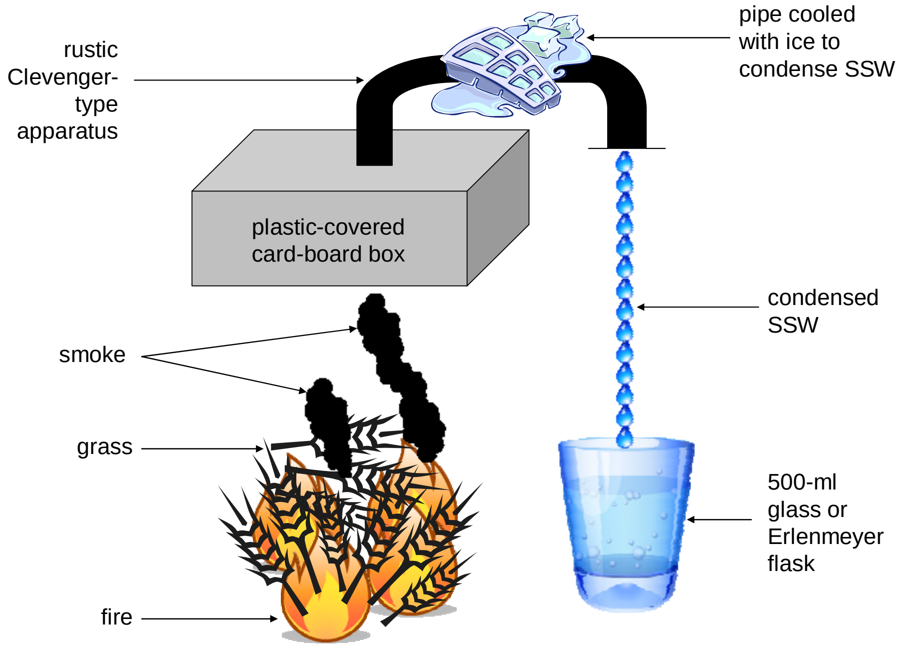

Fig. 1. Schematic of the experimental system for deriving smoke-saturated water (SSW) from grasses growing in Japan for *in vitro* experiments. Each grass is burned separately. To 1 kg of plant material (aerial, flowering parts), 100 ml of tap water is sprayed to increase the level of steam. As smoke rises and is captured by a plastic-covered card-board box, it travels up a pipe 10 cm in diameter. As the smoke reaches the U-turn in the pipe, which is cooled externally by packets holding blocks of ice or crushed ice, the hot steam condenses and runs down into a glass vessel to capture the SSW. This is the "concentrate" that is then diluted down to 1, 5 or 10% (v/w) in TC medium.

Results and discussion

The most notable finding of this study is that SSW could not stimulate *neo*-PLB formation more than the control treatment containing PGRs (standard TC medium), and performed as poorly as (with 1% SSW) or worse than (5 or 10% SSW) the control treatment without PGRs (Fig. 2). The latter concentrations tended to "bleach" explants (Fig. 3C), suggesting some negative impact on the photosynthetic apparatus. In this study, unlike other studies in which SSW or smoke has improved seed germination, SSW has had an inhibitory effect. This is not altogether a bizarre result since the seed germination of many species has not been stimulated by SSW (e.g., BROWN & al. 2003; SPARG & al. 2005; ZHOU & al. 2013), in the latter study only one out of 13 species in the southern tropical belt of China (Guangzhou) being stimulated by a commercial Australian-derived SSW. Even though PLBs are analogous to somatic embryos, and even though somatic embryos are analogous to zygotic embryos (i.e., within seed), it is evident that either a) SSW would also not stimulate hybrid *Cymbidium* seed, or that b) the mechanism by which SSW impacts

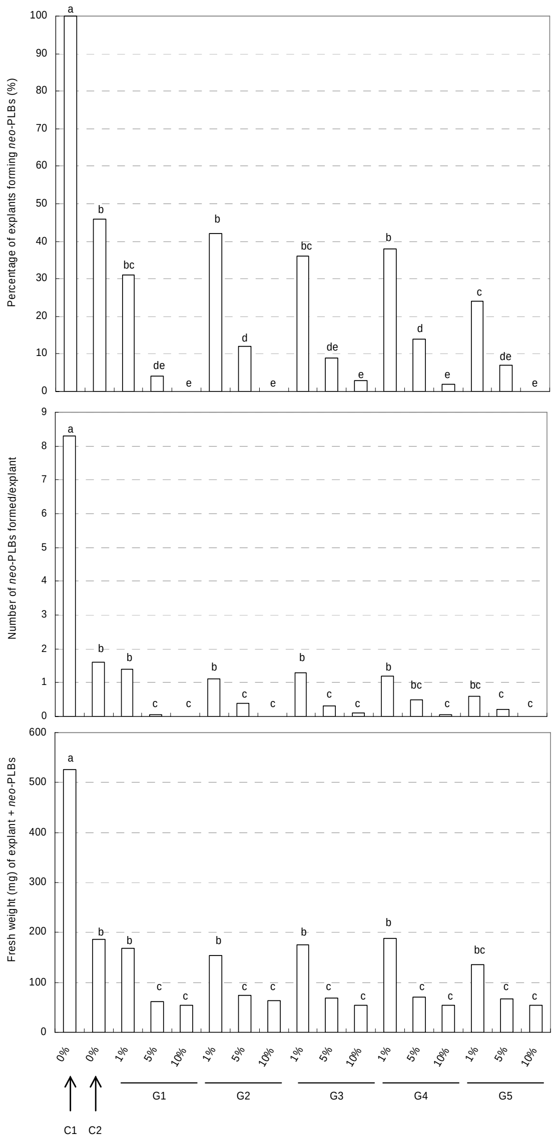

Fig. 2. Impact of smoke-saturated water (SSW) from five grasses growing in Japan on *neo*-PLB formation from hybrid *Cymbidium* Twilight Moon 'Day Light' half-PLBs after 60 days in culture [TEIXEIRA DA SILVA & DOBRÁNSZKI, 2013]. Three parameters were assessed: (Top) Percentage of half-PLBs forming *neo*-PLBs (%)*; (Center) Number of *neo*-PLBs formed per half-PLB; (Bottom) Fresh weight (mg) of half-PLB + *neo*-PLBs**. Notes: * All percentage data was arc-sine transformed prior to analysis. ** average fresh weight of initial half-PLB explants is 54 mg (n = 10). Treatment notes: Control 1 = TC + PGRs (0.1 mg/l NAA + 0.1 mg/l Kin); Control 2 = TC – PGRs; G1 = *Arundinella hirta* (Thunb.) C. Tanaka var. *hirta*; G2 = *Microstegium japonicum* (Miquel) Koidzumi; G3 = *Miscanthus sinensis* Andersson; G4 = *Paspalum thunbergii* Kunth ex Steud.; G5 = *Themeda triandra* Forssk. var. *japonica* (Willd.) Makino. Mean values with by the same letter are not significantly different based on DMRT (P = 0.05). n = 90 (10 × 3 × 3). C = control; G = grass species; Kin, kinetin; NAA, α-naphthaleneacetic acid; PGR, plant growth regulator; PLB = protocorm-like body; TC = Teixeira *Cymbidium* medium No. 1 [TEIXEIRA DA SILVA 2012].

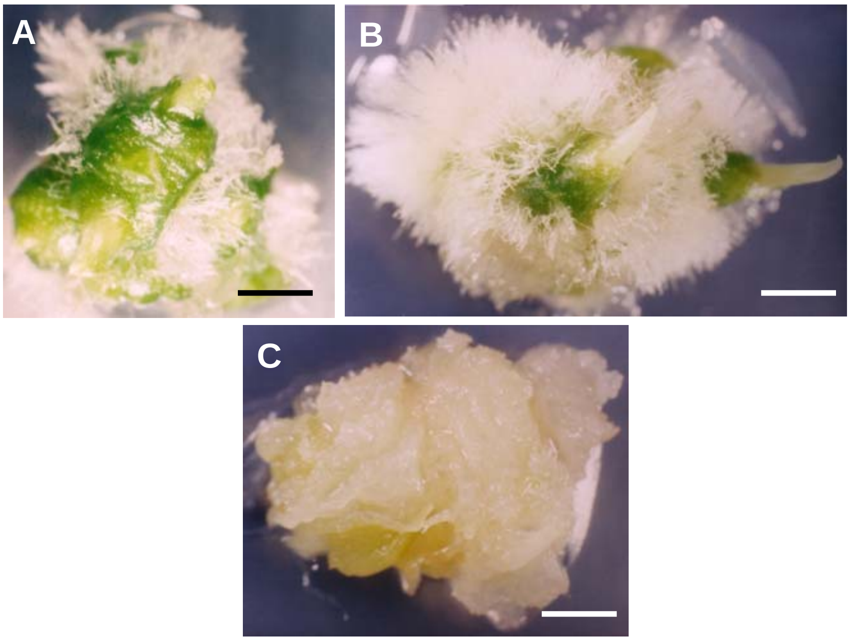

Fig. 3. Growth and development of hybrid *Cymbidium* Twilight Moon 'Day Light' *neo*-PLBs under control (no SSW) (A), 1% (v/w) *Themeda triandra* Forssk. var. *japonica* (Willd.) Makino SSW (B), or 5% *Microstegium japonicum* (Miquel) Koidzumi SSW (C) in solid basal TC [TEIXEIRA DA SILVA 2012] medium. Bars = 1 mm.

seed germination and *neo*-PLB formation is different. Most likely the latter option is more plausible since a component of the mechanism underlying smoke-induced germination is gibberellin synthesis, which results in a decrease in abscisic acid pools [GARDNER & al. 2001; SCHWACHTJE & BALDWIN, 2004].

A search of the literature did not reveal any study on the use of SSW derived from plants in Japan (endemic, or not) on seed germination, or on other plant-based responses. One of the possible reasons may be that the climate is tropical to sub-tropical in the south and south-west, becoming increasingly temperate as one moves north. The incidence of bush fires in Japan is rare and rainfall is generally abundant, so one would imagine that vegetation – specifically seed germination – has evolved to survive without the need for fire, unlike in Mediterranean-style climates like in Western Australia [ROCHE & al. 1994], the South African Cape fynbos [BAXTER & al. 1994], or the US California chaparral [KEELEY & FOTHERINGHAM, 1997]. Even so, the seed of not all plants are sensitive to smoke or SSW, displaying family-wide divergence [BROWN & al. 2003; SPARG & al. 2005; ZHOU & al. 2013]. Hybrid *Cymbidium* could generally be referred to as broadly tropical, requiring high humidity to survive and propagate effectively, and fire would not be a natural form of asymbiotic seed germination. Even though SSW has been shown to effectively stimulate seed germination in some orchids (*Vanda parviflora*, *Xenikophyton smeeanum*, *Pholidota pallida*, *Oberonia ensiformis*) [MALABADI & al. 2008, 2011, 2012; MULGUND & al. 2012], to date no study has examined the use of SSW on *in vitro* organ development of other orchids. However, two other studies have shown that SSW stimulates somatic embryogenesis in *Baloskion tetraphyllum* [MA & al. 2006] and *Brassica napus* [ABDOLLAHI & al. 2012]. No other studies on the use of SSW for assessing *in vitro* plant organogenesis appear to have been conducted. This area of research is at a nascent phase of development and many more trials would be required on more plant species, including horticultural, medicinal and agronomic, to assess the broad range of effects *in vitro* and under greenhouse and field trials. Key questions that still need to be answered: A) What is the toxicity of SSW, as assessed by toxicity assays? B) What is the mechanism by which a plant takes up and responds to SSW? C) To what level and in what organelles and parts of the plant are SSW or components of SSW accumulated, or used? The fact that each batch of grass that is burnt would likely yield a different concentration of active compounds in the SSW is a weakness of the protocol. Yet, by using only one compound such as butenolide or KAR would most likely not be a viable solution to this problem since SSW may contain multiple organogenesis-influencing factors or PGR-like substances other than the KAR family.

Conclusions

Smoke-saturated water (SSW) can provide a valuable alternative to conventional plant growth regulators for stimulating *in vitro* or *in situ* growth. In this study, SSW derived from five grass species growing in Japan inhibited protocorm-like body formation in hybrid *Cymbidium*, which would not be unusual considering that fire or smoke are not aspects related to this genus under natural/wild conditions.

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