Induction of adventitious shoots and tetraploids in *Antirrhinum majus* L. by treatment of antimitotic agents in vitro without plant growth regulators

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**Abstract**  We examined the effects of five antimitotic agents using *Antirrhinum majus* L. ‘Maryland True Pink’ on the induction of adventitious shoots resulted in increase of frequencies of chromosome doubling without plant growth regulators. Seeds were treated in vitro with 0, 16.5, 32.9, 65.8, 131.6, or 263.2 µM oryzalin (ORY), amiprofos-methyl (APM), butamifos (BUT), or prophan (IPC) or 800, 1,600, 3,200, 6,400, or 12,800 µM colchicine (COL) for 7 day. ORY, COL and APM promoted induction of adventitious shoots on the hypocotyls at maximum frequencies of 57.6% with 16.5 µM ORY, 5.6% with 800 µM COL and 88.8% with 131.6 µM APM. ORY and COL also induced adventitious shoots on the epicotyls adjacent to the cotyledons, particularly at high concentrations, with a maximum frequency of 26.0% at 12,800 µM COL. APM treatment increased frequencies of tetraploids from 0.0 to 93.1%, with a positive correlation between the frequency and concentration. By contrast, ORY and COL induced tetraploids at frequencies of 16.0 to 54.6% and 4.0 to 59.4%, respectively, with peaks at both low and high concentrations of each. Correlation analysis revealed that frequencies of adventitious shoot formation could be useful as an index for the induction of tetraploids. These results showed that three of the antimitotic agents tested induced both adventitious shoot and tetraploid without plant growth regulators, indicating that antimitotic action may play a common role in the induction of adventitious shoot.

**Key words:** adventitious shoots, antimitotic agents, *Antirrhinum majus* L., chromosome doubling, epicotyl/hypocotyl.

*Antirrhinum majus* L. (2n = 2x = 16) is a popular ornamental plant in the family Plantaginaceae that is widely cultivated. Consequently, the main objective in breeding this species is to develop cultivars with novel horticultural characteristics, such as flower color, flower longevity, and resistance to biotic or abiotic stresses (Çelikel et al. 2010; Horn 2002).

Polyploidization has been reported to provide novel and attractive traits with greater marketability, as well as increased resistance to biotic or abiotic stresses (Aina et al. 2012; Dhooghe et al. 2011; Mori et al. 2016; Nimura et al. 2006; Nonaka et al. 2011; Thao et al. 2003; Van Laere et al. 2011), and the development of polyploid plants can be induced artificially through the application of antimitotic agents (Dhooghe et al. 2011). Colchicine (COL), an alkaloid, is the most commonly used antimitotic agent to induce polyploidy through chromosome doubling (Hancock 1997). However, several other antimitotic agents have also recently been shown to successfully produce polyploids in vitro, such as oryzalin (ORY), amiprofos-methyl (APM), and trifluralin (de Carvalho et al. 2005; Dhooghe et al. 2009; Hansen and Andersen 1996; Khosravi et al. 2008; Kondo et al. 2020; Nimura et al. 2006; Nonaka et al. 2011; Rey et al. 2002).

The induction of adventitious shoots from segments of hypocotyl cultured on medium supplemented with plant growth regulators (PGRs), such as auxin and cytokinin, has been reported in many plant species (Basalma et al. 2008; Ghnaya et al. 2008; Kim et al. 1997; Makunga and Van Staden 2008; Rai 2002), including *A. majus* (Busse et al. 2005; Cui et al. 2004; Okubo et al. 1991). Furthermore, Kamada and colleagues successfully induced the development of embryos from carrot (*Daucus carota*) seedlings without any PGRs by applying stress(es), i.e., high concentrations of sucrose (Kamada et al. 1993), salt solutions (Kiyosue et al. 1989), heavy metals ions (Kiyosue et al. 1990) and high temperatures (Kamada et al. 1994). Ikeda-Iwai et al. (2003) successfully induced somatic embryogenesis in *Arabidopsis thaliana* by applying stress(es) such as osmotic, heavy metal ions, and dehydration with 2,4-dichlorophenoxyacetic acid.

We previously revealed using an herbicide
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Tokunol-M® (Bayar Crop Science, Tokyo, Japan) containing 60.0% (w/w) APM that successfully induced adventitious shoots on the hypocotyls of A. majus seedlings in vitro without any PGRs. These adventitious shoots developed into tetraploids with a maximum frequency of 100% (Hlaing et al. 2020).

Among the various antimitotic agents, ORY and trifluralin are categorized as dinitroaniline herbicides, COL as a plant alkaloid, APM and butamifos (BUT) as phosphoric amide herbicides, and propham (IPC) and chlorpropham as carbamates. However, all these chemicals are recognized as metaphase inhibitors and disrupt the microtubule spindles or microtubule-organizing centers, resulting in the inhibition of chromosome separation during metaphase (Dhooghe et al. 2011; Hidalgo et al. 1989; Hugdahl and Morejohn 1993; Morejohn et al. 1987a, b; Rost and Morrison 1984). Therefore, the aims of the present study were to examine whether ORY, COL, IPC, and BUT, beside APM can induce adventitious shoots and/or chromosome doubling in A. majus when applied in vitro without any PGRs, as previously observed for APM using a herbicide Tokunol-M® (Hlaing et al. 2020). The induction of adventitious shoot was then discussed in terms of whether they are independent or share, in part, as antimitotic agents, a common mechanism.

Seeds of the F1 cultivar A. majus ‘Maryland True Pink’, which is one of the leading cultivars in the cut flower industry, were used in this experiment. Raw seeds without any pretreatment after the harvest were purchased from Miyoshi & Co., Ltd. (Tokyo, Japan) and kept at 4°C in an airtight container with silica gel for 1–6 weeks before use.

Seeds were sterilized and sown in vitro, as described by Hlaing et al. (2020) unless stated otherwise. The following antimitotic agents were obtained at standard grades for pesticide residue analysis: APM from Hayashi Pure Chemical Industries Ltd. (Osaka, Japan); ORY (3, 5-dinitro-N4, N4-dipropylsulfanilamide), BUT (O-ethyl O-6-nitro-m-tolyl sec-butylphosphoramidothioate), IPC (isopropyl carbanilate) and COL from Fujifilm Wako Pure Chemical Industries, Ltd. (Osaka, Japan).

Table 1. Effects of various concentrations of five antimitotic agents on the percentage survival and frequency of tetraploid induction in A. majus L. ‘Maryland True Pink’.

| Antimitotic agents | Concentrations (µM) | Frequency of survival percentage (%) | No. of plants analyzed by FCM | Number of plants with different ploidy level | Frequency of tetraploid induction (%) |
|--------------------|---------------------|--------------------------------------|------------------------------|----------------------------------------------|--------------------------------------|
|                    |                     |                                      |                              | 2x                                           |                                      |
|                    |                     |                                      |                              | 4x                                           |                                      |
|                    |                     |                                      |                              | 8x                                           |                                      |
|                    |                     |                                      |                              | 2x+4x                                        |                                      |
|                    |                     |                                      |                              | 4x+8x                                        |                                      |
| ORY                | 0                   | 91.2 abc                             | 60                           | 60                                           | 0                                    | 0.0 d                                |
|                    | 16.5                | 41.2 ij                              | 62                           | 4                                            | 27                                   | 16 8 7 43.6 bc                       |
|                    | 32.9                | 22.8 kl                              | 44                           | 6                                            | 24                                   | 10 2 2 54.6 bc                      |
|                    | 65.8                | 0.8 m                                | 0                             | 0                                            | 0                                    | 42.6 b                              |
|                    | 131.6               | 18.8 kl                              | 25                           | 10                                           | 4                                    | 4 5 2 16.0 d                        |
|                    | 263.2               | 49.2 hi                              | 54                           | 12                                           | 23                                   | 5 10 4 42.6 bc                      |
| COL                | 0                   | 93.2 a                               | 60                           | 60                                           | 0                                    | 0 0 0.0 d                           |
|                    | 800                 | 72.4 efg                             | 101                          | 14                                           | 60                                   | 5 11 11 59.4 b                      |
|                    | 1,600               | 77.6 cdef                            | 78                           | 22                                           | 31                                   | 0 24 1 39.7 c                       |
|                    | 3,200               | 76.4 defg                            | 100                          | 72                                           | 7                                    | 0 21 0 7.0 d                        |
|                    | 6,400               | 68.4 fg                              | 90                           | 44                                           | 4                                    | 42 0 4.0 d                          |
|                    | 12,800              | 63.2 gh                              | 76                           | 13                                           | 40                                   | 11 8 52.6 bc                       |
| APM                | 0                   | 91.6 abc                             | 51                           | 51                                           | 0                                    | 0 0 0.0 d                           |
|                    | 16.5                | 90.8 abc                             | 94                           | 92                                           | 0                                    | 0 2 0 0.0 d                         |
|                    | 32.9                | 76.4 defg                            | 67                           | 34                                           | 10                                   | 0 23 0 14.9 d                       |
|                    | 65.8                | 38.4 ij                              | 52                           | 3                                            | 45                                   | 1 0 3 86.5 a                        |
|                    | 131.6               | 27.6 jk                              | 27                           | 1                                            | 25                                   | 0 1 0 92.6 a                        |
|                    | 263.2               | 12.0 lm                              | 29                           | 1                                            | 27                                   | 0 1 93.1 a                         |
| IPC                | 0                   | 89.2 abcd                            | 60                           | 60                                           | 0                                    | 0 0 0.0 d                           |
|                    | 16.5                | 90.0 abcd                            | 100                          | 100                                          | 0                                    | 0 0 0.0 d                           |
|                    | 32.9                | 88.4 abcd                            | 100                          | 100                                          | 0                                    | 0 0 0.0 d                           |
|                    | 65.8                | 84.4 abcd                            | 100                          | 100                                          | 0                                    | 0 0 0.0 d                           |
|                    | 131.6               | 83.4 abcd                            | 100                          | 100                                          | 0                                    | 0 0 0.0 d                           |
|                    | 263.2               | 64.4 fg                              | 80                           | 80                                           | 0                                    | 0 0 0.0 d                           |
| BUT                | 0                   | 92.0 ab                              | 80                           | 80                                           | 0                                    | 0 0 0.0 d                           |
|                    | 16.5                | 86.8 abcd                            | 100                          | 100                                          | 0                                    | 0 0 0.0 d                           |
|                    | 32.9                | 85.2 abcd                            | 100                          | 100                                          | 0                                    | 0 0 0.0 d                           |
|                    | 65.8                | 87.2 abcd                            | 100                          | 100                                          | 0                                    | 0 0 0.0 d                           |
|                    | 131.6               | 87.2 abcd                            | 100                          | 100                                          | 0                                    | 0 0 0.0 d                           |
|                    | 263.2               | 78.0 bcdef                           | 100                          | 100                                          | 0                                    | 0 0 0.0 d                           |

Values within the same column that are followed by different letters are significantly different at p<0.05 (Tukey's test).
APM, BUT, and IPC were prepared as 500 µM stock solutions, while COL was prepared as a 16.7 mM stock solution. Ethanol was used as a solvent at a concentration of 0.08 to 1.3%. All stock solutions were freshly prepared and filter-sterilized before use.

The seeds were submerged in 300-ml Erlenmeyer flasks that contained 250–280 seeds each in 10 ml of an aqueous solution of 16.5, 32.9, 65.8, 131.6, or 263.2 µM ORY, APM, BUT, or IPC; 800, 1,600, 3,200, 6,400, or 12,800 µM COL; or water as a control. The flasks were then shaken at 40 rpm at 20°C under continuous light of 20 µmolm⁻²s⁻¹ for 7 days. After treatment, the seeds were rinsed 5 times with sterilized distilled water and 50 seeds from each treatment were transferred to a Petri dish (90 mm diameter, 20 mm deep) containing 20 ml of Murashige and Skoog (MS) medium without any PGRs that had been solidified with 2.5 g l⁻¹ Gellan gum (Fujifilm Wako Pure Chemical Industries, Ltd., Osaka, Japan). The cultures were kept at 20°C under a 16-h light period, using a cool white inflorescent light at an intensity of 70 µmolm⁻²s⁻¹ during the light period. All seedlings were sub-cultured every 4 weeks after treatment with each antimitotic agent. Each treatment was replicated five times.

The frequencies of induced adventitious shoots on the mother seedlings were recorded at 31 days after the initiation of culture on MS medium and the frequencies of surviving seedlings were recorded after 8 weeks. The frequency of surviving seedlings for each treatment included both surviving mother seedlings and plantlets derived from the adventitious shoots on the mother seedlings that showed severe necrosis and designated as dead.

The youngest expanded leaves (ca. 3–5 mm in length) were collected from the first node of each seedling in vitro and analyzed by FCM, following the methods of Kondo et al. (2020) unless stated otherwise.

The significance of differences among treatments was analyzed by analysis of variance followed by Tukey's test. In the tables that follow, values within the same column that are followed by different letters are significantly different at p<0.05. Relationship between the frequencies of seedlings induced adventitious shoots and tetraploid induction were assessed by correlation coefficient analysis. The index used by Kondo et al. (2020) for the efficiency of tetraploid induction was modified and calculated as follows: Efficiency of tetraploid induction index = frequency of germination of seeds (%) × frequency of survival (%) × frequency of tetraploids (%).

The frequencies of surviving seedlings in the water-treated control groups ranged from 89.2 to 93.2% (Table 1). ORY treatment resulted in all the mother seedlings dying at all concentrations examined, so frequencies of surviving seedlings were only obtained when adventitious shoots replaced the mother seedlings. Seedlings survived with a frequency of 41.2% following treatment with 16.5 µM ORY, which decreased to 0.8% with 65.8 µM ORY but then increased again to 49.2% with 263.2 µM ORY (Table 1). The frequencies of surviving seedlings slightly decreased from 72.4 to 63.2% as the concentration of COL increased from 800 to 12,800 µM. APM treatment reduced the frequencies of surviving seedlings to 12.0% from 90.8%, with survival decreasing with an increasing concentration of APM. Furthermore, mother seedlings that received 65.8–263.2 µM APM stopped growth and were replaced by plantlets derived from adventitious shoots on the hypocotyls (Figure 1A).

The frequencies of surviving seedlings decreased from 90.0 to 64.4% and 86.8 to 78.0% as the concentrations of IPC and BUT, respectively, increased from 16.5 µM to 263.2 µM (Table 1). All the seedlings that established from seeds treated with IPC or BUT at any concentration were almost identical in morphology to those in the water-treated control group.

All of the antimitotic agents examined induced changes in the morphology of the seedlings in a concentration-dependent manner. In addition, three of the five antimitotic agents (i.e., ORY, COL and APM) induced adventitious shoots on the hypocotyls and/or epicotyls (Table 2, Figure 1). In the water-treated control group, only normal seedlings established in vitro.

All of the seedlings that established from seeds...
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Table 2. Effects of various concentrations of five antimitotic agents on the frequencies of adventitious shoot on the hypocotyls and epicotyls of *A. majus* L. 'Maryland True Pink'.

| Antimitotic agents | Concentrations (µM) | Frequencies of seedlings induced adventitious shoot on | | |
|--------------------|---------------------|------------------------------------------------------|-------|
|                    |                     | Hypocotyl (%)                                          | Epicotyl (%) | Total (%) |
|                    |                     | Single adventitious shoot | More than one adventitious shoot | | |
| ORY                | 0.0                 | 0.0 f | 0.0 e | 0.0 d | 0.0 g |
|                    | 16.5                | 48.0 a | 9.6 cd | 0.0 d | 57.6 c |
|                    | 32.9                | 43.2 ab | 9.6 cd | 0.0 d | 52.8 cd |
|                    | 65.8                | 4.0 ef | 0.0 e | 0.0 d | 4.0 fg |
|                    | 131.6               | 21.6 d | 4.4 de | 2.0 cd | 28.0 e |
|                    | 263.2               | 30.0 cd | 2.8 de | 10.0 b | 32.8 d |
| COL                | 0                   | 0.0 f | 0.0 e | 0.0 d | 0.0 g |
|                    | 800                 | 5.6 ef | 0.0 e | 4.8 c | 10.4 fg |
|                    | 1,600               | 0.4 f | 0.0 e | 0.0 d | 0.4 fg |
|                    | 3,200               | 0.0 f | 0.0 e | 0.0 d | 0.0 g |
|                    | 6,400               | 0.0 f | 0.0 e | 0.0 d | 0.0 g |
|                    | 12,800              | 0.0 f | 0.0 e | 0.0 d | 0.0 g |
| APM                | 0.0                 | 0.0 f | 0.0 e | 0.0 d | 0.0 g |
|                    | 16.5                | 11.6 e | 0.0 e | 11.6 f |
|                    | 32.9                | 65.8  | 43.2 ab | 26.4 b |
|                    | 131.6               | 51.2 a | 37.6 a | 0.0 d | 88.8 a |
|                    | 263.2               | 34.8 bc | 32.8 c | 0.0 d | 67.6 cd |
| IPC                | 0.0                 | 0.0 f | 0.0 e | 0.0 d | 0.0 g |
|                    | 16.5                | 11.6 e | 0.0 e | 11.6 f |
|                    | 32.9                | 65.8  | 43.2 ab | 26.4 b |
|                    | 131.6               | 51.2 a | 37.6 a | 0.0 d | 88.8 a |
|                    | 263.2               | 34.8 bc | 32.8 c | 0.0 d | 67.6 cd |
| BUT                | 0.0                 | 0.0 f | 0.0 e | 0.0 d | 0.0 g |
|                    | 16.5                | 11.6 e | 0.0 e | 11.6 f |
|                    | 32.9                | 65.8  | 43.2 ab | 26.4 b |
|                    | 131.6               | 51.2 a | 37.6 a | 0.0 d | 88.8 a |
|                    | 263.2               | 34.8 bc | 32.8 c | 0.0 d | 67.6 cd |

Values within the same column that are followed by different letters are significantly different at *p* < 0.05 (Tukey’s test).

Treated with ORY experienced inhibition of growth of the seminal roots but exhibited swollen hypocotyls and cotyledons and eventually produced adventitious shoots on some hypocotyls. There were two peaks in the frequencies of seedlings with adventitious shoots on the hypocotyls and epicotyls among the concentrations examined. The frequencies of adventitious shoots reached a maximum of 57.6% for seedlings that received 16.5 µM ORY, decreased to 4.0% with 65.8 µM, and then increased again to 28.0% with 131.6 µM ORY and 42.8% with 263.2 µM ORY (Table 2, Figure 1B). However, the induced adventitious shoots on the hypocotyls of seedlings that received 65.8–263.2 µM ORY stopped growing and ultimately died.

The seedlings that were treated with 263.2 µM ORY could be separated into three groups based on their germination velocities. In the first group, the seeds germinated early, and the plants eventually died, with no induction of adventitious shoot. In the second group, the seeds germinated later and produced one adventitious shoot per hypocotyl at frequency of 30% and more than one adventitious shoot per hypocotyl at a frequency of 2.8% (Table 2) but did not show further growth and eventually died. In the third group, the seeds germinated later still and produced adventitious shoots on the epicotyls, which survived. The frequency of adventitious shoots on the epicotyls reached 10.0% with 263.2 µM ORY and more than one adventitious shoot was produced per epicotyl, whereas the maximum frequency of seedlings with more than one adventitious shoot on the hypocotyls was 9.6% with the 16.5 µM and 32.9 µM ORY treatments but only 2.8% with 263.2 µM ORY (Table 2).

The frequency of adventitious shoots on the hypocotyls decreased as the concentration of COL increased, with values of 5.6% with 800 µM COL and 0% with 12,800 µM (Table 2), and growth of these adventitious shoots was suppressed so they did not grow further. By contrast, the frequency of adventitious shoots on the epicotyls adjacent to the cotyledons increased as...
the concentration of COL increased, reaching 26.0% with 12,800 µM COL (Table 2, Figure 1C).

Seedlings that established from seeds treated with 16.5 µM and 32.9 µM APM exhibited slightly swollen hypocotyls that developed adventitious shoots at frequencies of 0% and 11.6%, respectively (Table 2). By contrast, seedlings that established from seeds treated with 65.8–263.2 µM APM exhibited intensely swollen hypocotyls that eventually produced adventitious shoots (Figure 1D, E). The frequencies of induction of adventitious shoots increased until 28–31 day after the initiation of culture on MS medium and reached a maximum value of 88.8% at a concentration of 131.6 µM (Table 2). No adventitious shoots developed on the epicotyls following APM treatment. An increasing concentration of APM increased not only the frequency of total seedlings that produced adventitious shoots but also the number of adventitious shoots on each mother seedling (Table 2, Figure 1E). Thus, APM induced one adventitious shoot per hypocotyl at frequencies of 11.6% at a concentration of 32.9 µM and 51.2% at 131.6 µM and induced more than one adventitious shoot per hypocotyl at frequencies of 0% at concentrations of 16.5–32.9 µM and 37.6% at 131.6 µM.

Seedlings that established from seeds treated with the highest concentrations of IPC and BUT showed minimal swelling of the hypocotyls in the early stages and grew in an almost identical way to seedlings in the water-treated control groups, with no induction of adventitious shoot (Table 2). ORY, COL and APM effectively induced the development of tetraploids, whereas IPC and BUT had no such effect (Table 1, Figure 2).

The frequencies of tetraploids among seedlings that established from seeds treated with 16.5 µM and 32.9 µM ORY were 43.6% and 54.6%, respectively (Table 1). No data were obtained from seedlings in the 65.8 µM ORY treatment group, as they were severely damaged and eventually died. However, seedlings that had been treated with 131.6 µM and 263.2 µM ORY produced adventitious shoots on the epicotyls adjacent to the cotyledons (Figure 1B) and eventually developed into tetraploids at frequencies of 16.0% and 42.6%, respectively.

Seedlings that established from seeds treated with the lowest concentration of COL (800 µM) produced tetraploids at a frequency of 59.4%, whereas the frequencies of tetraploids decreased to 7.0% with 3,200 µM COL and 4.0% with 6,400 µM COL, with no induction of adventitious shoot (Table 1). However, 52.6% of seedlings in the 12,800 µM COL treatment group were found to be tetraploids, which was almost the same as for the 800 µM COL treatment group.

The frequency of tetraploid induction increased with an increasing concentration of APM. Frequency of tetraploids ranged from 0.0 to 93.1% among seedlings in the 16.5–263.2 µM APM treatment groups.

ORY and APM treatments showed the significant positive correlation between the frequencies of seedlings induced adventitious shoots and those of tetraploids (rAPM = 0.93, p = 0.006; rORY = 0.96, p = 0.002, respectively). A moderate positive correlation between these two parameters was also observed in COL treatment (rCOL = 0.71, p = 0.11).

We previously reported that the herbicide Tokunol-M™ (Bayer Crop Science, Tokyo, Japan), which contains 60.0% APM, induced adventitious shoots in the absence of PGRs in A. majus, which developed into tetraploids (Hlaing et al. 2020). In the present study, we found that the application of APM at a standard grade for pesticide residue analysis showed almost identical tendency in frequencies of induction of adventitious shoots and tetraploid development as were obtained in our previous study with Tokunol-M™ (Tables 1, 2).

Antimitotic agents such as COL, ORY, and trifluralin in combination with PGRs have previously been shown to promote development of somatic embryos in zygotic embryo cultures of Ilex paraguariensis (Rey et al. 2002), while COL in combination with osmotic stress and PGRs promoted embryogenesis from microspores in rice anther culture (Ferreres et al. 2019). In an investigation of chromosome doubling in trihaploid kiwifruit (Actinidia delicosa), Chalak and Legave (1996) reported that the promotion of adventitious shoots regeneration from leaf explants increased by ca. 26% when the concentration of ORY increased from 5 to 15 µM, whereas COL did not induce adventitious shoot at any of the concentrations examined.

Kamada and colleagues reported that certain types of stress(es) can induce somatic embryogenesis in carrot and A. thaliana, such as osmotic stress (sucrose, mannitol, NaCl, etc.), heavy metal ions (Cd²⁺, Fe³⁺, etc.), heat shock, and dehydration (Harada et al. 1990; Ikeda-Iwai et al. 2003; Kamada et al. 1993, 1994; Kiyosue et al. 1989, 1990). Ikeda-Iwai et al. (2003) also proposed that stress(es) could induce a common reaction that is linked to plant cell de-differentiation and re-differentiation and is probably involved in the induction of somatic embryogenesis. Our previous study revealed that APM has a dual effect, inhibiting the growth of mother seedlings and stimulating the induction of adventitious
shoots on the hypocotyls of seedlings that developed into tetraploids (Hlaing et al. 2020). The antimitotic agents that were used in this study have been shown to disrupt the microtubule spindles (Hugdahl and Morejohn 1993; Morejohn et al. 1987a, b; Strachan and Hess 1983) or microtubule-organizing centers, inhibiting normal chromosome separation during metaphase (Hidalgo et al. 1989). Thus, the stress(es) induced by the loss of microtubule function, cellular structure and abnormal chromosome separation could be involved in the regulation of adventitious shoot induction on the hypocotyls and/or epicotyls of seedlings, though further experiments will be required to elucidate this.

While all the antimitotic agents used in this study have a similar mode of action, their chemical structures and plant tubulin binding affinities differ (Hugdahl and Morejohn 1993). Furthermore, and plant tubulin binding affinities differ (Hugdahl and Morejohn 1993). Thus, the stress(es) induced by the antimitotic agents used in this study have been shown to disrupt the microtubule spindles (Hugdahl and Morejohn 1993; Hugdahl and Morejohn 1993). Furthermore, Murthy et al. (1994) demonstrated that APM binds with tobacco (Nicotiana tabacum) tubulin with a moderate affinity, with an estimated value of 15 µM compared with 5.6 µM for ORY. However, in the present study, the maximum frequencies of adventitious shoot induction were greatest for APM (88.8%), followed by ORY (57.6%) and COL (26.0%) and thus did not correlate with the degree of affinity for tubulin shown in previous studies. One possible explanation for this is that the optimum concentrations for the induction of adventitious shoots and tetraploids development could differ among different species and/or target units, such as cells, tissues, and organs.

In the present study, the morphological responses of the seedlings differed according to not only the concentrations of the antimitotic agents used but also the germination velocity of the seeds, as observed for the three groups of seedlings in the 263.2 µM ORY treatment group. Zavattieri et al. (2010) reported that the response of wounded cells or tissues to stress(es) depends on both the level of stress applied and the physiological stage of the cells. Therefore, we postulate that the physiological stage/condition of each seed that received treatment differed to some extent, resulting in different morphological responses of the seedlings to the applied stress. ORY and COL stimulated adventitious shoot formation on the epicotyls, particularly at higher concentrations, whereas APM had no such effect (Table 2, Figure 1B, C). Potters et al. (2007) argued that, in general, the morphological responses of plants to sub-lethal stress(es) include the inhibition of cell elongation, localized stimulation of cell division, and alteration in the cell differentiation status. Thus, the mode of action of stress(es) induced by treatment with ORY and COL may be different from those induced by APM, eventually inducing adventitious shoots on the epicotyls at certain concentrations. Furthermore, it is possible that the regions in which antimitotic agents induce adventitious shoots may depend on the outcome of competition between cells in the hypocotyl and epicotyl, which varies according to the concentration applied. Therefore, further experiments are needed to reveal the mechanism that regulates the position of occurrence of adventitious shoots.

In the present study, BUT and IPC did not induce adventitious shoot on the hypocotyls or epicotyls (Table 2). Ikeda-Iwai et al. (2003) reported that the source of stress, concentration of stress chemicals, and duration of exposure are important factors for the induction of somatic embryogenesis in A. thaliana. Thus, the concentrations of these two antimitotic agents may have been below the threshold for inducing adventitious shoot on the hypocotyls of seedlings, as the hypocotyls showed only minimal swelling in seedlings that established from seeds treated with the highest concentrations of these chemicals.

Treatments with higher concentrations of the antimitotic agents, such as 65.8 to 263.2 µM APM, caused the mother seedlings to die and be replaced by the adventitious shoots (Table 1, Figure 1A). There are two possible explanations for this: 1) once adventitious shoots had been induced, growth of the adventitious shoots was less inhibited by the antimitotic agent due to its turnover by the tissues; or 2) the induced adventitious shoots were more resistant to the inhibitory effects of the antimitotic agent than the original seedlings. We previously reported that the induction and subsequent development of adventitious shoots depended on mortality of the mother seedlings and the rate of induction of adventitious shoots (Hlaing et al. 2020). In the present study, we observed that the induced adventitious shoots on the hypocotyls did not grow and died if early mortality of the mother seedlings occurred, particularly at higher concentrations of APM and ORY (Tables 1, 2). Thus, it seems likely that subsequent development of the adventitious shoots depends on sustainment of the mother seedlings, which may play a role in turnover of the antimitotic agents, supporting the first possibility outlined above. However, chronological analyses of the concentrations of the antimitotic agents in the cells or tissues will be required to confirm this.

Those treatments that induced adventitious shoot on either the hypocotyls or epicotyls also induced high frequencies of tetraploid development (Tables 1, 2). Correlation analysis indicated that there is a strong positive relationship between these two parameters. Therefore, we hypothesize that the abiotic stress caused by ORY, COL and APM was a prerequisite for the induction of adventitious shoots and chromosomes doubling and resulted in high frequencies of tetraploid...
adventitious shoots. Possibly, antimitotic action of these agents may play a common role in the induction of adventitious shoots and chromosome doubling in *A. majus*.

Kondo et al. (2020) recently proposed the index of chromosome doubling to evaluate the appropriate combination of APM concentrations, exposure time, and the efficiency of chromosome doubling. We modified this index to show the frequency of tetraploid based on the seeds treated in vitro. We found that treatment with 800 µM COL showed the highest frequencies of tetraploid (index - 39.2%), followed by 65.8 µM APM (32.6%) and 263.2 µM ORY (19.5%). The maximum tetraploid indexes which were expected to be obtained from the seeds sown were not significant difference between COL and APM. These findings support those of previous studies, whereby APM and COL have almost identical in maximum chromosome doubling efficiencies but an approximately 100 times lower concentration of APM is required to achieve this (Hansen and Andersen 1996; Hansen et al. 1998). Although the efficiency of chromosome doubling by antimitic agents has been shown to differ among plant species (Dhooghe et al. 2011), APM and ORY have been found to be more efficient in several species (Dhooghe et al. 2009; Nimura et al. 2006; Nonaka et al. 2011; Sakhanokho et al. 2009; Sree Ramulu et al. 1991; Stanys et al. 2006), while COL has also been reported to have high efficiency (Alan et al. 2007; Eeckhaut et al. 2002; Petersen et al. 2002, 2003; Podwyszyńska et al. 2017; Rey et al. 2002; Wu and Mooney 2002). Therefore, it is possible that the binding affinity and concentrations of APM applied in this study were optimal for the induction of tetraploids in *A. majus*.

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