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SHORT COMMUNICATION

Doubling effect of anti-microtubule herbicides on the maize haploid

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ABSTRACT

Doubled haploid (DH) technology is widely used in crop breeding programs. Colchicine is the most frequently used chemical agent in the DH inducing. However, colchicine has disadvantage of high poisonousness. It is necessary to find some non-toxic or low toxic substitutes that should have the same effect as colchicine. In this study, two anti-microtubule herbicides, amiprophosmethyl (AMP) and Oryzalin, were used to double the haploids under different treatment conditions, and the doubling effects were compared with colchicine. The results showed that the highest doubling rate induced by APM is about 45% in the condition of 20 μM 24h. We calculated the actual doubling rate (survival rate × double rate), 38.23%, 20.64%, and 19.4% are the highest actual doubling rates induced by APM, colchicine, and oryzalin, respectively. Although both APM and Oryzalin can induce maize haploid doubling, comparing with the actual doubling rate, APM is better than colchicine. In addition, soaking seed had been proved the best way to operate and obtain the highest doubling haploids in all conditions. As a low toxic mitotic inducer, APM is a good substitute of colchicine in doubling maize haploids, which is suitable for application in the DH-based breeding pipeline.

Keywords: Maize; Anti-microtubule; Haploid; Doubling effect

INTRODUCTION

Haploid Plants, which contain only one complete set of chromosomes can be spontaneously or artificially, induced chromosome doubling to form "doubled haploids" (DH) (Arshadullah et al., 2018; Ong et al., 2017). In maize breeding, using DH technology breeders can shorten the breeding cycles and develop completely homozygous lines in 2–3 generations, compared with the conventional inbred line development process, which takes at least 6–8 generations to derive lines with ~99% homozygosity (Geiger and Gordillo, 2009; Chang and Coe, 2009). Haploid induction has been recognized worldwide as an important measure for enhancing breeding efficiency and been adapted by commercial maize breeding programs, with maize haploid inducers available in breeding practice. (Schmidt, 2003; Seitz, 2005; Chen et al., 2009; Prigge and Melchinger, 2011; Kebede et al., 2011).

Chromosome doubling is necessary for maize haploids to restore fertility. Studies showed that colchicine can inhibit mitotic and induce chromosome doubling (Chase, 1969; Deimling et al., 1997). A scholar reported that 18% of haploid seeds were chromosome doubled by treated 12h with 0.06% colchicine (Davarnejad et al., 2018; Jiang et al., 2018; Khaleel et al., 2018; Sankarganesh et al., 2018). The fertility rate reached to 61% (Seaney, 1955) in roots treatment with colchicine. Although colchicine can induce chromosome doubling, it is not completely effective, because sectoral diploidization of inflorescences can occur. In addition, it has a disadvantage of high poisonousness (Stadler, 1989). Other herbicides, with less toxic (Geiger and Gordillo, 2009), such as pronamide, amiprophos methyl (APM), trifluralin, and Oryzalin, were also efficient mitotic inhibitors (Hantzschel and Weber, 2010). A scholar found that four different kinds of herbicide, APM, pronamide, Oryzalin and trifluralin, could cause chromosome doubling. Colchicine, Oryzalin and APM have similar function as colchicine by inhibiting the formation of microtubules in culture of anther of Brassica napus (Hansen et al., 1996). A scholar found that the doubling rate was about 11.05% by treated wheat with APM. APM treatment caused the mitotic index of onion meristem from 0.8% to 5.2% at mitotic metaphase (Zhen-ying, 2003).

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Anti-microtubule herbicides are lower toxic and more effective than colchicine in chromosome doubling in plant (Li et al., 2018; Wang et al., 2018). However, few studies are about haploid doubling by treated with anti-microtubule herbicides in maize. In order to choose a low toxic and effective haploid doubling reagent, two kinds of anti-microtubules, AMP and Oryzalin, and colchicine were used to treat seeds, buds, and shoots of maize haploids in different concentrations at different growth time points (Howlader et al., 2018; H'ng et al., 2018; Fahim and Sathi, 2018). Moreover, the results of this study may provide the theory basis and feasible measure for improving the technology of haploid doubling in maize.

MATERIALS AND METHODS

Maize haploids used in this research were induced from a hybrid (Zheng 58 × PH6WC) by the haploid inducer, JS6-2. We set three treatment groups, the seed immersion group, the bud immersion group, and the root immersion group. In each treatment, 50 putative haploid progenies were treated and planted at the Breeding Station of Jilin agricultural university, Changchun, China. The stock solution of APM or Oryzalin is 3mM, which were mixed with 0.1% Tween-20 and 2% DMSO. Final concentrations (10μM, 15μM, 20μM, 25μM, and 30μM) were adjusted by ddH2O. Colchicine with the concentration of 0.5 mM, 1.0 mM, 1.5 mM, and 2.0 mM were prepared in 0.1% Tween-20 and 2% DMSO and dissolved in ddH2O, respectively. For the seed immersion, the haploid seeds were soaked in water for 6 hrs at room temperature, and then cut slightly around the embryo. The cut seeds were immersed in the different agents (AMP, Oryzalin, or Colchicine) at 28° for 8h, 16h, 24h, and 48h. Where after they were rinsed with water for 30 minutes. For the bud immersion, the seeds were soaked in water at room temperature for 6 hrs. and germinated on a wet germination paper at 28°. After the roots had grown to about 1cm, the temperature was lowered to 24° in order to make the buds to germinate. When the shoots grew to about 2 cm, we cut the top 3mm of the coleoptiles, which were soaked at 28° in the treatment agents for 8h, 16h, 24h, and 48h. Later they were soaked in water for 30 min and then grown in nursery site at greenhouse (28°) before they grew to 4 or 5 leaf-age which can be transplanted to field. For the root immersion, the seeds were soaked in water at room temperature for 6 hrs. and placed in an incubator at 28° until the roots grew up to 2 cm. They were placed in the 4-8° condition for 3 hrs. Then, the roots were immersed in the agents he treatment agents for 8h, 16h, 24h, and 48h before transplanted to the field. All three treatments were replicated 3 times, respectively.

The chromosome ploidy level of induced progenies was checked by Flow cytometry (FCM). When the third leaf emerged, the tip of second leaf was cut off, chopped into small pieces and stained with 2 ml DAPI (4'6-diamidino-2-phenylindone) solution (5 μg/ml, Partec GmbH, Germany), filtered through a nylon membrane (50 μm mesh size). The filtrate was analyzed by FCM, at a par gain FL1 (fluorescence) of 420-430 nm (relative fluorescence-RF). The peak of DH should be 60-130 FL. In contrast, the peak of haploid plant should be 30-70 FL (Dang et al., 2012).

RESULTS

In order to compare the doubling effect of haploids by using different ways, the survival rate and doubling rate of haploids were analyzed. Under the three different chemical treatments, the results of survival rate of haploids showed that survival rates obviously decreased with the increase of the concentration and the processing time in the most kinds of treatment groups (Fig 1). Especially, in each groups of haploid seeds treated by APM, the survival rates were not significant different, which indicated the toxic effects of APM to haploid seeds was limited and not obviously enhancing with the concentration increasing.

Comparing the different approaches, the survival rate in the seed-soaked group was significantly higher than the others. This result indicated that root tips and bud tips were more sensitive to the chemical treatments. And the mortality rates increased significantly with the concentration increasing in the bud/root treated groups. Soaking seeds is not only easy to operate but also suitable to get more survival seedlings, which is better than soaking bud/root ways.

To compare the doubling effect of three chemicals, the double rates have been analyzed. The results showed that APM was better than the other two (Fig 2). The highest doubling rate induced by colchicine is about 30% in the condition of 1.5mM 24h; the highest doubling rate induced by APM is about 45% in the condition of 20 μM 24h; and highest doubling rate induced by oryzalin is about 32% in the condition of 25 μM 24h. We calculated the actual doubling rate (survival rate × double rate), 38.23%, 20.64%, and 19.4% are the highest actual doubling rates induced by APM, colchicine, and oryzalin, respectively. We also found that all the highest doubling rates in our research were induced in the soaking seed groups.

Comprehensively analyzing various Doubling methods based on the average value of haploid doubling rates, we

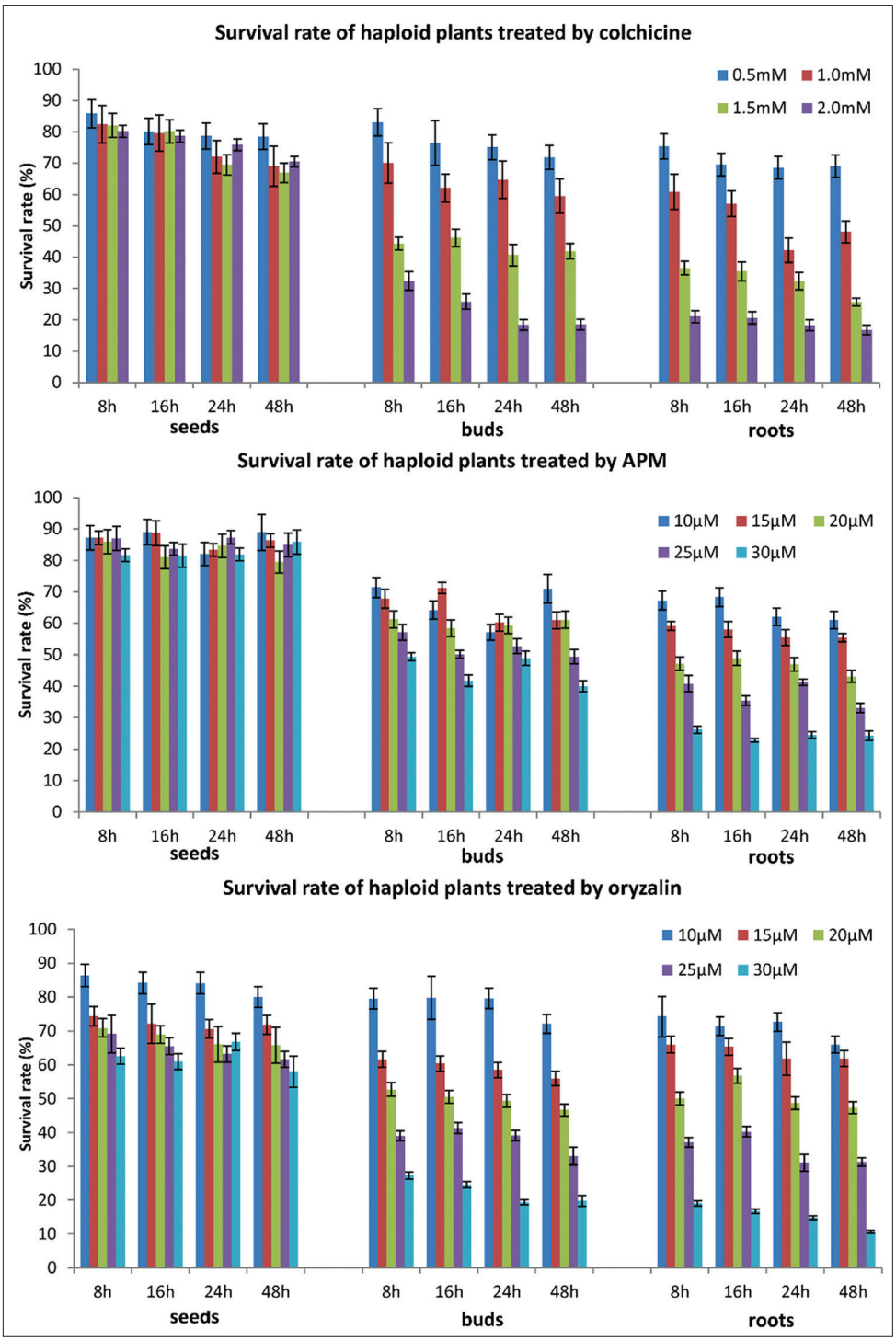


Fig 1. Survival rate of maize haploids treated by colchicine, APM, and oryzalin.

clearly found, APM is the best to induce haploid doubling in the three kinds of agents, while soaking seed not only has the advantage of easy operation, but also had obtained the significantly higher doubling effect than the other ways (Table 1). In this study, the best haploid doubling way was the haploid seeds were soaked in the 20μM of APM for 24h. Using colchicine, the best way was to soak seeds in the 1.5 mM of colchicine for 24h; Using ORYZALIN, the best way was to soak seeds in the 25μM of ORYZALIN for 24h.

DISCUSSION

Many studies have proved that the maize lines which come from haploid doubling are completely homozygous and homogeneous. Doubled haploid lines have the

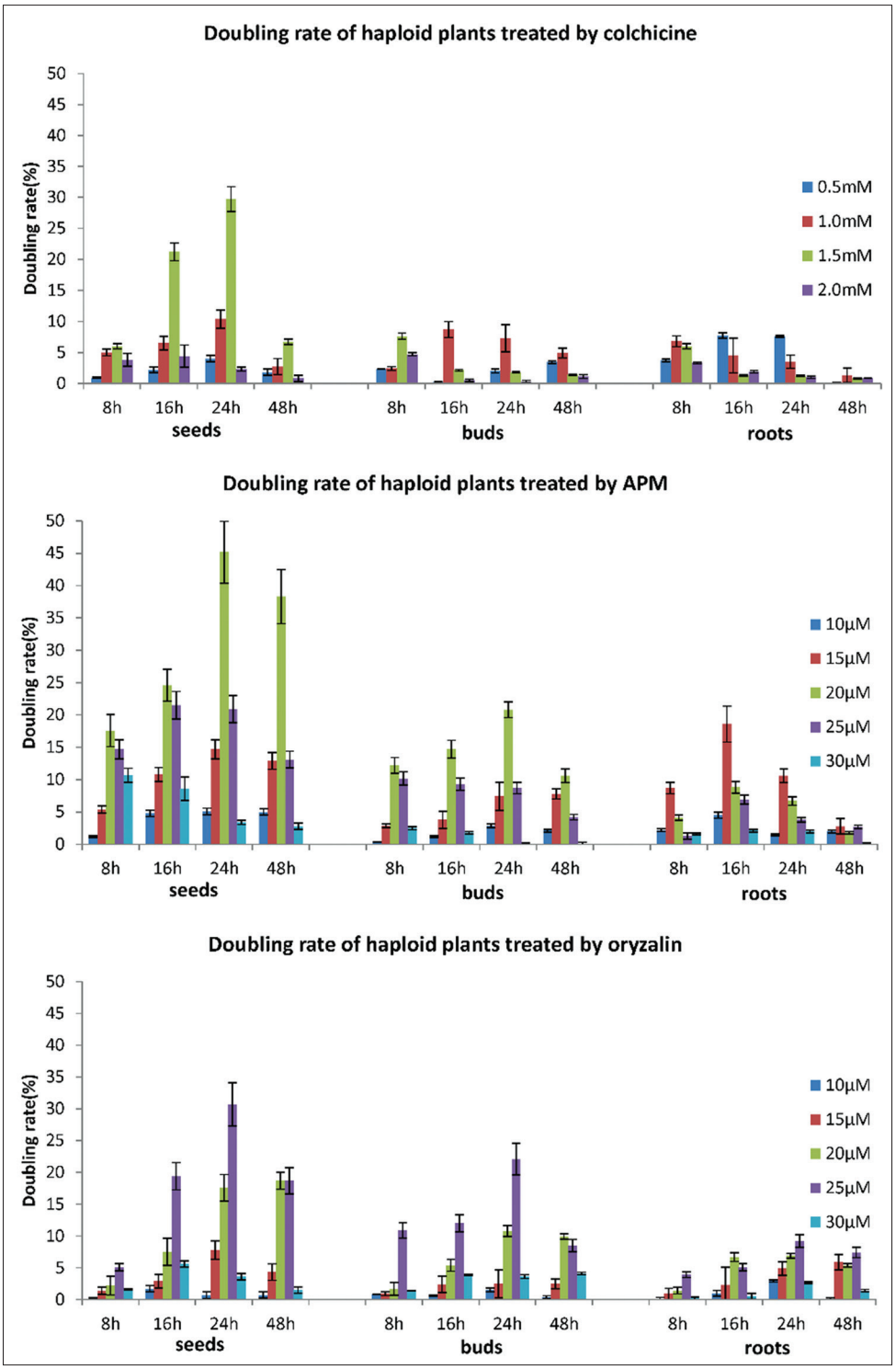


Fig 2. Doubling rate of maize haploids treated by colchicine, APM, and oryzalin.

advantage of increasing the efficiency of selection, reducing the breeding cycles, and decreacing the effort for line maintenance (Chang and Coe, 2009; Geiger and Gordillo, 2009; Röber et al., 2005; Geiger, 2009). Our studies showed that the spontaneous doubling rate was very low at only about 1.5%. Colchicine is most frequently used for inducing haploid doubling. In this study, the highest doubling rate of colchicine was 29.7%, which was remarkably higher than the spontaneous haploid doubling rate. Other studies on colchicine were similar to ours. The highest doubling rate of colchicine treatment in Wen's study was 48.35%, while a scholar's results showed that the highest doubling rate was about 23% (Liu, 2000). Another study showed that a haploid doubling rate of 19.8% appeared with treatment in six-leaf-stage by colchicine (Wei, 2007).

Table 1: Comparison of the doubling effect under the different treatments

	Doubling rate					
	Seed soaked		Bud soaked		Root soaked	
Colchicine	5.10%	Ba	1.77%	Cb	1.68%	Bb
APM	11.82%	Aa	3.60%	Ab	2.35%	Ac
Oryzalin	5.00%	Ba	2.23%	Bb	1.58%	Bc

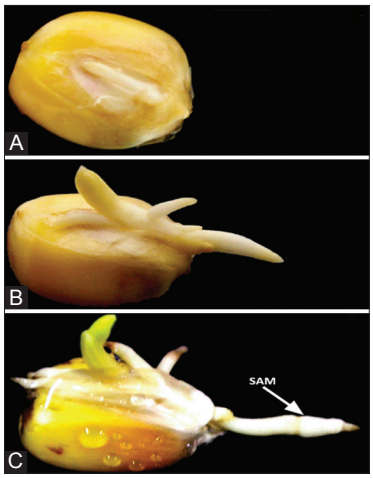
A,B,C present the significant difference between the agents; a,b,c presents the significant difference between the treated organs (P<0.01)

Although colchicine was used to induce haploid doubling widely, it has disadvantage of high poisonousness (Stadler, 1989). It is necessary to find some non-toxic or low toxic substitutes that should have the same effect as colchicine. Some herbicides, such as pronamid, amiprophosmethyl (APM), trifluralin, and Oryzalin, are good candidates, because they are not only efficient mitotic inhibitors but also less toxic compared to colchicine (Geiger and Gordillo, 2009; Hantzschel and Weber, 2010). In our study, APM and Oryzalin were selected to research the effect of inducing haploid doubling in maize.

Compared with Oryzalin and colchicine, the survival rate under APM treatment was higher. The results indicated that the inhibitory action of APM was weaker than oryzalin and colchicine in maize seedling development. A previous study found that APM can effectively double the chromosomes of callus tissue with no toxic effect on regenerated plants (Wan et al.1991). Similar results also showed that APM did not inhibit "Black Mexican Sweet" callus culture at 50 µM for 21-28 hrs. Oryzalin also has an effect on doubling haploid. Some researchers found that callus regeneration was decreased severely at high concentration of Oryzalin, while a low concentration did not double chromosome efficiently (Duncan and Widholm, 1989). In our study, Oryzalin treatment was found to have a lower survival rate compared with the other two methods, although the doubling rate was better than colchicine.

Our results showed that both APM and Oryzalin can induce maize haploid doubling. However, comparing the actual doubling rate, just APM is better than colchicine. In addition, soaking seed had been proved to be the best way to operate and obtain the highest doubling haploids in all conditions. In this stage, a large number of cells in haploid seeds are in the period of mitosis, which makes them more likely to be treated by chemical agents.

We observed the morphological changes of the haploid seeds throughout the treatment process. No growth was observed during the process of soaking seed. In the recovery process, the soaked seeds resumed normal growth, and SAM (shoot apical meristems) regions were observed in most of soaked seeds (Supplementary Fig 1). The same phenomenon was reported in a previous study (Hantzschel et al., 2010).



Supplementary Fig 1. Examples of morphological changes in the treatment A, a seed soaked. B, a seed in the growth recovery; C, a SAM region in the soaked seed.

In conclusion, in all methods of doubling haploids, soaking seeds with APM showed that the best results, although all three chemical agents, APM, Oryzalin, and colchicine, have the effect of inducing haploid doubling. In our research, the highest doubling rate induced by APM is about 45% in the condition of 20 µM 24h. As a low toxic mitotic inhibitor, APM is a good substitute to colchicine in doubling maize haploids, which is suitable for application in the DH based breeding pipeline.

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