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The fungal plant pathogen *Microsphaeropsis amaranthi* is virulent against a number of key weeds in the Amaranthaceae, including common waterhemp, and is under investigation as a bioherbicide. Common waterhemp has become a key weed in midwestern crop production systems and is a good target for a bioherbicide that could be integrated into weed management systems. We investigated the direct effects of a range of chemical herbicides and adjuvants upon conidia of *M. amaranthi* and found that many herbicides and most adjuvants were strongly inhibitory to germination. On the other hand, *M. amaranthi* was compatible with a selection of postemergence herbicides commonly used in midwestern weed management systems, including carfentrazone, chloransulam, and imazethapyr. Most glyphosate products suppressed or abolished germination of *M. amaranthi* conidia, but by testing adjuvants commonly used in glyphosate products and technical-grade glyphosate salts, it was revealed that this inhibition was due to formulation additives and not the active ingredient. When glyphosate and conidia of *M. amaranthi* were sprayed onto common waterhemp seedlings, the herbicide predisposed plants to infection by *M. amaranthi*. When *M. amaranthi* was applied 1 to 3 d after glyphosate, the glyphosate rate required to control common waterhemp was reduced by half. Similar results were observed on clones propagated from a common waterhemp plant resistant to glyphosate. When *M. amaranthi* was applied to seedlings 2 d before glyphosate, the efficacy of the herbicide was reduced. These findings demonstrate that positive interactions between herbicides and *M. amaranthi* exist but reveal practical difficulties that may limit the integration of the strategy in the field.

Nomenclature: *Microsphaeropsis amaranthi* (Ell. & Barth.); common waterhemp, *Amaranthus rudis* Sauer, AMATA.

Key words: Bioherbicide, common waterhemp, interaction, integrated weed management, glyphosate.

Common waterhemp has become a key weed in the midwestern United States as a result of its suitability to no-till farming practices (Cordes et al. 2004; Hartzler et al. 2004) and the development of an array of herbicide-resistant biotypes, some of which are widespread in the region (Li et al. 2004; Patzoldt et al. 2002, 2005; Shoup et al. 2003). Resistance of common waterhemp to glyphosate is under investigation (Smeda 2000; Smith and Hallett 2006; Zelaya and Owen 2005). Glyphosate has become the predominant postemergence herbicide used in soybean [*Glycine max* (L.) Merr.] in the midwestern United States, with approximately 80% of soybean acres sown to glyphosate-resistant varieties (Duke 2005).

The fungal plant pathogen *Microsphaeropsis amaranthi* (Ell. & Barth.) is a candidate for development as a bioherbicide. It is easily cultured, grows rapidly, and sporulates profusely on a number of artificial media (Smith 2003). In addition, it is host-restricted to the Amaranthaceae (Mintz et al. 1992) and virulent to a number of important weeds in the genus *Amaranthus* (Mintz et al. 1992; Ortiz-Ribbing and Williams 2006; Smith and Hallett 2003).

It is a challenge to develop host-restricted bioherbicides, such as *M. amaranthi*, for the control of weeds of major agricultural or horticultural crops because they only control one, or a few, weed species within a weed community of multiple species. Consequently, a particular need for the investigation of a host-restricted bioherbicide must be established before project initiation and a means for integration of the bioherbicide into weed management systems must be established during product development (Charudattan 2001; Hallett 2005). The interactions between bioherbicides and chemical herbicides have been reviewed by Altman et al. (1990); Christy et al. (1993); Greaves and Sargent (1986); Hoagland (1996), and Lévesque and Rahe (1992). Many positive interactions between bioherbicides and chemical herbicides have been demonstrated (Léger et al. 2001; Smith and Hallett 2003; Wymore et al. 1987). Sharon et al. (1992) and Lévesque and Rahe (1992) showed that sublethal doses of glyphosate can inhibit the ability of plants to produce phytoalexins and thus defend themselves against pathogens.

The need and opportunity for the development of a host-restricted bioherbicide for common waterhemp stems from its unusually dominant status in midwestern cropping systems. Whereas most annual broadleaf weeds are effectively managed by current herbicide-based approaches, common waterhemp is a frequent escapee and often represents a single weed problem following weed management interventions, particularly in midwestern soybeans (Cordes et al. 2004; Hartzler et al. 2004). This situation may be exacerbated if growers continue to rely heavily upon glyphosate for postemergence weed control and the efficacy of gly-

phosate on common waterhemp further declines under this selection pressure. Consequently, a market need and opportunity may exist for the development of a bioherbicide targeting common waterhemp. The present study was designed to evaluate the opportunities and limitations to the integration of the *M. amaranthi* bioherbicide into production systems by testing the direct effects of chemical herbicides and adjuvants on the germinability of conidia and evaluating the interactions between glyphosate and *M. amaranthi* on common waterhemp.

Materials and Methods

Production of Plants and Fungal Inoculum and Spray Application

Seed of common waterhemp was pretreated by soaking with gentle agitation (1.5 × g, 18 h, 20 C) in 0.14mM gibberellic acid (GA_3), then sown onto a commercial potting mix,[1] and maintained in the greenhouse. *M. amaranthi*[2] was recovered from soil cultures and maintained on V8 agar (Singleton et al. 1982). Conidia were harvested from young colonies (10 to 14 d) for each experiment by scraping with a sterile cotton swab, filtering through four layers of cheesecloth, and adjusting conidial concentrations with the aid of a hemacytometer.[3]

A glyphosate-resistant common waterhemp plant from a population in Altamont, IL (ALT 1),[4] and a susceptible plant from a population in Fowler, IN (FOW 1) (Smith and Hallett 2005) were selected to compare the interactions of glyphosate and *M. amaranthi* applied as split applications. To maintain the genotypes of these plants—common waterhemp is dioecious—we propagated clonal populations. Plants were kept in a vegetative state by repeated pruning, repotting, and fertilizing and by maintaining a day length of 16 h with supplemental lighting. Shoot apices that were approximately 25 mm in length were cut just below axillary buds, dipped in rooting hormone (Rootone),[5] planted into fresh potting soil, and maintained in a humid environment under a clear-plastic cover for 7 d in the greenhouse. In this way, numerous clones of each selected parent plant were generated. Plastic covers were removed from propagated plants at least 48 h before treatment. The vegetative propagation technique provided a clonal population of uniform size.

Suspensions of *M. amaranthi* at 3×10^6 conidia ml^{-1} were applied using XR8003 nozzle tips[6] at 300 kPa of pressure and a spray volume of 548 L ha^{-1} in a commercial spray chamber. Unless otherwise stated, plants were moved to a dew chamber[7] immediately after treatment, where they were maintained with constant leaf wetness (18 h, dark, 100% relative humidity [RH], 18 C) until being returned to the greenhouse. Glyphosate[8] was applied as 0.63 kg ae ha^{-1} using XR8003 spray tips at 300 kPa pressure and a spray volume of 186 L ha^{-1}.

Direct Effects of Chemical Herbicides and Adjuvants

Tank-mix experiments were designed to simulate the impact of herbicides and adjuvants upon *M. amaranthi* in the spray tank environment. The herbicides selected are all commonly used postemergent herbicides for corn (*Zea mays* L.) or soybean production in the midwestern United States. Conidia of *M. amaranthi* (1.5×10^6 conidia ml^{-1}) were incubated in solutions of 30 different chemical herbicides or adjuvants (Table 1) in sterile cell-culture plates (15 mm) on an orbital shaker for 2 h. The concentrations of chemical herbicides and adjuvants used were 0, 0.25×, 0.5×, 1×, 2×, and 4× (× = recommended label rate). After 2 h of incubation, conidial germination was stimulated by the addition of 1 ml of half-strength potato dextrose broth to each plate, and germination was counted under a compound microscope using four views of 25 conidia for each of four replicates after a further 3 h incubation on the shaker. The experiment was arranged in a randomized complete-block design, was performed twice, and the data from the two trials were combined, following a test for homogeneity of variances (Gomez and Gomez 1984), then regressed using PROC REG in SAS.[9] The values of the concentration causing a 50% reduction in germination (GR_{50}) were calculated from regression equations.

Timing of Glyphosate and *M. amaranthi* Applications

Common waterhemp seedlings (three- to four-leaf stage) were sprayed with glyphosate or conidia of *M. amaranthi* or both, at the following relative timings: (1) water control, (2) conidia alone, (3) glyphosate alone, (4) conidia and glyphosate mixed and incubated for 2 h before application, (5) glyphosate sprayed first followed immediately (approx. 10 min) by conidia, (6) glyphosate sprayed first followed by conidia 1 d later, (7) glyphosate sprayed first followed by conidia 3 d later, and (8) conidia sprayed first followed by glyphosate 2 d later. Plants were placed in the dew chamber, as described above, immediately after application of conidia, but were not placed in the dew chamber after glyphosate applications. In the case of the tank mixture (treatment 4) and the 10-min split application (treatment 5), plants were placed in the dew chamber as per conidial applications. Plant mortality and aboveground dry weights were measured 10 d after the later spray application. Sprays were staggered to ensure that plants were all harvested at the same age. The experiment was arranged in a randomized complete-block design, performed twice, and the data from the two trials combined. Plant mortality and dry weights were analyzed by ANOVA using PROC GLM in SAS, with means separated using Duncan's multiple range test, and graphed as a percentage of the control in Sigmaplot.[10]

Concentration of Glyphosate in Split Applications

Common waterhemp seedlings (three- to four-leaf stage) or clones (8 to 10 leaves) were sprayed with glyphosate or conidia of *M. amaranthi* or both as described above. Plants were sprayed with glyphosate first, then returned to the greenhouse for 24 h before application of conidia. Following the application of conidia, plants were maintained in a dew chamber as described above. Glyphosate treatments were prepared from a mixture of a technical-grade solution of the IPA salt of glyphosate and a solution of the formulation blank of Glyphomax Plus[8] to ensure that the concentrations of adjuvants remained constant as glyphosate concentrations were varied. The experiment was arranged in a randomized complete-block design, performed twice, and the data from

TABLE 1. Effect of selected chemical herbicides and adjuvants on the germination of conidia of *Microsphaeropsis amaranthi.*

Trade name	Active ingredient, comment on formulation[a]	GR_{50}[b,c]
Chemical herbicides:		
Aatrex 4L	Atrazine, SC	<0.25
Aim	Carfentrazone, EC	>4.0
Beacon	Primisulfuron, WG	0.9
Buctril	Bromoxynil, EC	<0.25
Cobra	Lactofen, EC	<0.25
Firstrate	Chloransulam, WG	2.1
Glyphomax Plus	Glyphosate IPA salt, SL, contains tallowamine surfactants	<0.25
Gramoxone	Paraquat, SL	0.6
Liberty	Glufosinate, SL	<0.25
Pursuit	Imazethapyr, SL	>4.0
Roundup Custom	Glyphosate IPA salt, no adjuvants	1.4
Roundup Original	Glyphosate IPA salt, contains tallowamine surfactants	<0.25
Roundup Ultramax	Glyphosate IPA salt, contains tallowamine surfactants	<0.25
Roundup Weathermax	Glyphosate K^+ salt, contains tallowamine surfactants	<0.25
Sencor	Metribuzin, WG	0.7
Touchdown	Glyphosate TMS salt, contains APG surfactants	0.3
Ultra Blazer	Acifluorfen, SL	1.2
Glyphosate Salts:		
Tech. grade K^+ salt	Glyphosate K^+ salt, no adjuvants	0.9
Tech. grade IPA salt	Glyphosate IPA salt, no adjuvants	0.8
Adjuvants:		
28 UAN		1.8
Activator 90	Nonionic surfactant	<0.25
AMS	Ammonium sulfate	3.2
APG 6206	Alkylpolyglucoside surfactant	0.8
Formulation blank of Glyphomax Plus	Constituents of Glyphomax Plus without glyphosate salt	<0.25
Herbimax	Crop oil concentrate	0.2
Silwet L-77	Organosilicone surfactant	>4.0
Toximol TA5	Tallowamine surfactant	<0.25
Toximol TA15	Tallowamine surfactant	<0.25
Triton X-100	Nonionic surfactant	<0.25
Tween 20	Anionic surfactant	3.7

[a] Codes for formulation type: EC, emulsifiable concentrate; SC, suspension concentrate; SL, soluble concentrate; WG, water dispersible granules.
[b] GR_{50} is the concentration that reduced germination of conidia of *M. amaranthi* by 50%.
[c] The concentration for each product is expressed as a proportion of the recommended concentration for that product.

the two trials were combined. Plant growth (dry weight 10 d after application of conidia) was analyzed by ANOVA using PROC GLM in SAS and means were separated using Duncan's multiple range test and graphed as a percentage of the control.

Results and Discussion

Direct Effects of Chemical Herbicides and Adjuvants

Incubation in solutions of chemical herbicides and adjuvants resulted in a range of impacts on the germination of conidia of *M. amaranthi* (Table 1). Germination was completely inhibited by many herbicides at concentrations tested, including most commercially available glyphosate herbicides, atrazine, bromoxynil, lactofen, glufosinate, and metribuzin. Germination was not affected, or only slightly reduced, by carfentrazone, chloransulam, imazethapyr, and glyphosate without adjuvants (Roundup custom). Most of the adjuvants tested either completely abolished or severely reduced germination of *M. amaranthi* conidia, with the exception of Silwett L-77 and Tween-20 (Table 1). Germination was inhibited by all formulated glyphosate products, particularly those containing tallow amine surfactants (Roundup Original, Roundup Ultramax, Roundup Weathermax, and Glyphomax plus), and was inhibited by solutions of tallow amine surfactants. Unformulated glyphosate products and technical grade glyphosate salts (Roundup custom, K^+ salt, and IPA salt) caused much lower levels of inhibition (Table 1).

The inhibition of germination of *M. amaranthi* conidia will affect the ability of the bioherbicide to be integrated into weed management systems in which these products are used. Each of the herbicides that severely affected germination are commonly used for the control of common waterhemp and other *Amaranthus* spp. in midwestern cropping systems (Loux et al. 2005). The inhibition of germination by a wide range of adjuvants used in chemical herbicides may also hamper efforts to integrate the bioherbicide into production systems. *M. amaranthi* was, however, stable in tank mixture with some postermergence herbicides, including carfentrazone, chloransulam, imazethapyr, and glyphosate without adjuvants.

The methodology used in this experiment was different from the methodology used by most researchers that have

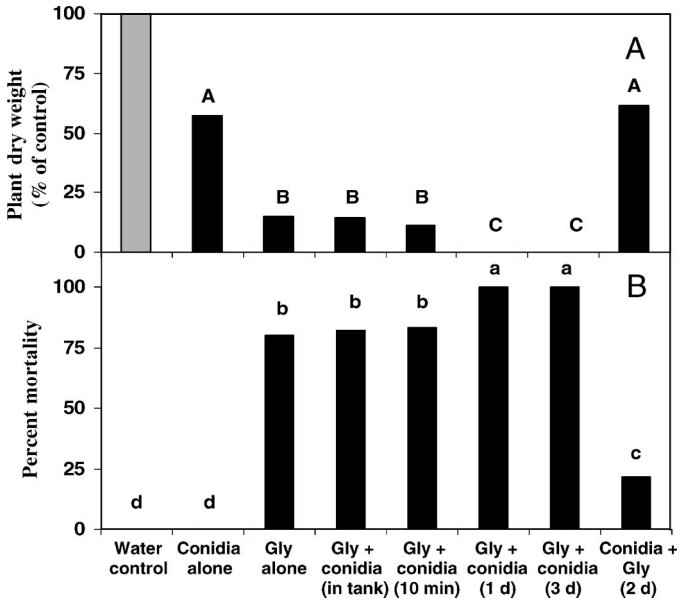

FIGURE 1. Effect of treatment of common waterhemp seedlings with glyphosate and conidia of *Microsphaeropsis amaranthi* at different times. (A) Plant dry weights 10 d after application. (B) Plant mortality 10 d after application. Bars with the same letter(s) are not significantly different (P = 0.05). Plant dry weights analyzed as a percentage of the water-treated control (value set to 100%; grey bar).

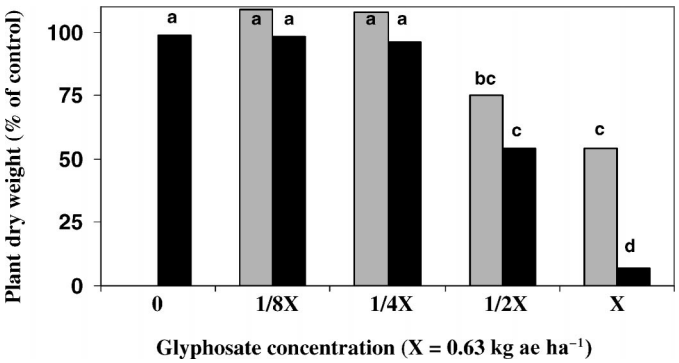

FIGURE 2. Dry weight of common waterhemp seedlings treated with glyphosate and *Microsphaeropsis amaranthi* in a split-application (glyphosate application followed by *M. amaranthi* 1 d later). Grey bars: glyphosate alone, Black bars: glyphosate plus *M. amaranthi*. Bars with the same letter(s) are not significantly different (P = 0.05).

measured the compatibility of bioherbicides with chemical herbicides. Whereas most researchers have incorporated test chemicals into agar (e.g., Wyss et al. 2004; Zhang et al. 2003), we incubated conidia directly in solutions of the test chemicals for 2 h. This was done specifically to simulate the environment of a spray tank during spraying of a field. In preliminary experiments (unpublished data), we found that these conditions were more severe than the agar method, presumably because the chemicals had been in contact with conidia for 2 h before the stimulation of germination by the addition of nutrients or because the chemicals are more accessible in liquid than in agar. We caution that the compatibility of bioherbicides with chemicals hitherto reported in the literature may be overestimated.

Timing of Glyphosate and *M. amaranthi* Applications

The interaction between glyphosate and *M. amaranthi* was strongly dependent upon the relative timing of the application. When glyphosate and conidia of *M. amaranthi* were applied as a tank mixture or applications were made within 10 min, plant mortality and dry weight reductions were not significantly different from applications of glyphosate alone (Figure 1). However, when applications of conidia were delayed for either 1 d or 3 d after glyphosate applications, plant mortality was increased to 100% (Figure 1). This is similar to the findings of Sharon et al. (1992) and Léger et al. (2001) who demonstrated predisposition of sicklepod (*Cassia obtusifolia* L.) and fireweed (*Epilobium angustifolium* L.), respectively, to bioherbicides by sublethal concentrations of glyphosate. We hypothesize that this interaction is caused by interference with the production of plant defense compounds (e.g., phytoalexins) due to inhibition of the shikimic acid pathway by sublethal concentrations of glyphosate as was demonstrated in the case of *C. obtusifolia* (Sharon et al. 1992). Tank mixtures and 10-min split applications showed no interaction between glyphosate and *M. amaranthi*, and we hypothesize that this was due to the direct toxic effects of glyphosate on conidia (Figure 1; Table 1).

Application of *M. amaranthi* and glyphosate in the reverse order—conidia applied 2 d before glyphosate—resulted in only 25% plant mortality, and dry weight reductions significantly lower than those caused by glyphosate alone (Figure 1). We propose two alternative hypotheses to explain this observation: (1) that developing infection foci of *M. amaranthi* in common waterhemp leaves altered source–sink relationships in the plant and reduced the translocation of glyphosate to meristems, or (2) that the proliferation of fungal mycelium and altered physical structure of leaf surfaces and epidermises reduced the absorption of glyphosate into the plant. We will investigate the mechanism of this inhibition in future studies.

Concentration of Glyphosate in Split Applications

Common waterhemp seedlings were only partially controlled by the recommended rate of glyphosate (× = 0.63 kg ae ha^{-1}), with dry weight reductions of approximately 50%, and the efficacy of lower concentrations was even less (Figure 2). The application of conidia of *M. amaranthi*, 1 d later, increased plant dry weight reductions to about 95% on plants pretreated with 1× glyphosate (Figure 2). The application of glyphosate to common waterhemp clones confirmed the susceptibility (FOW 1) and resistance (ALT 1) of our biotypes to glyphosate. Whereas 90% dry weight reductions of FOW 1 were caused by a 2× concentration of glyphosate, only an 80% dry weight reduction of ALT 1 was caused by an 8× concentration of glyphosate (Figure 3). The effect of subsequent applications of conidia of *M. amaranthi* had a similar impact in each clone, causing additional dry weight reductions at all concentrations of glyphosate. In the case of FOW 1, the dry weight reduction caused by a 2× concentration of glyphosate was not significantly different from that caused by a 1× concentration of glyphosate followed by *M. amaranthi* (Figure 3A). In the case of ALT 1, dry weight reductions caused by 4X or 8X concentrations of glyphosate were not significantly different

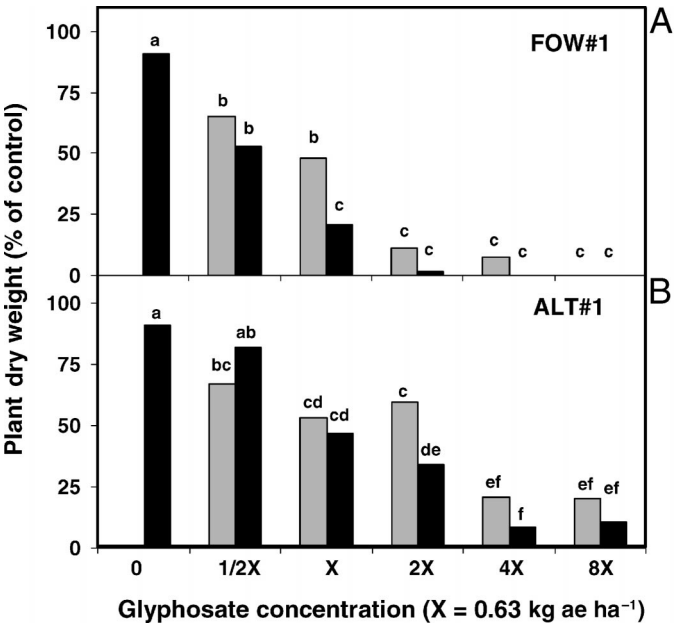

FIGURE 3. Dry weight of common waterhemp clones from populations (A) susceptible to glyphosate (FOW 1) and (B) resistant to glyphosate (ALT 1) treated with glyphosate and *Microsphaeropsis amaranthi* in a split-application (glyphosate application followed by *M. amaranthi* 1 d later). Grey bars: glyphosate alone, Black bars: glyphosate plus *M. amaranthi*. Bars with the same letter(s) are not significantly different (P = 0.05).

from the dry weight reductions caused by a 2× concentration of glyphosate followed by *M. amaranthi* (Figure 3B).

The response of common waterhemp to glyphosate is highly variable (Smith and Hallett 2006; Zelaya and Owen 2005), and poor control in the field is not uncommon. The application of *M. amaranthi* consistently increased the levels of control of common waterhemp by glyphosate. The efficacy of the recommended rate of glyphosate was increased on seedlings and FOW 1 clones, and the effective dose of glyphosate on ALT 1 clones was reduced from greater than 4× to 2×. These findings are important, given the current dominance of glyphosate-tolerant crops because they indicate that *M. amaranthi* may have value as a niche product by improving the efficacy of common waterhemp control in weed management systems using glyphosate.

Sources of Materials

[1] Metro Mix 360, Hummert International, 4500 Earth City Expressway, Earth City, MO 63045.

[2] Culture of *Microsphaeropsis amaranthi* kindly donated by Prof. G. Weidemann, Department of Plant Pathology, University of Arkansas, Fayetteville, AR 72701.

[3] Hemacytometer. Fisher Scientific, 1 Liberty Lane, Hampton, NH 03842.

[4] Common waterhemp plant from a population in Altamont, IL, kindly donated by Dr. C. Sprague, Department of Crop and Soil Sciences, Michigan State University, East Lansing, MI 48824.

[5] Rootone: TechPac LLC, PO Box 24830, Lexington, KY 40504.

[6] TeeJet, 3062 104th Street, Urbandale, IA 50322.

[7] Dew Chamber, Percival Scientific, 505 Research Drive, Perry, IA 50220.

[8] Glyphosate used in experiments employing a fixed rate was Glyphomax Plus®, Dow AgroSciences LLC, 9330 Zionsville Rd, Indianapolis, IN 46268. Where glyphosate concentrations were varied, separate components viz. technical-grade glyphosate (IPA salt) and formulation blank of Glyphomax Plus® were mixed to ensure concentrations of adjuvants remained constant. Components provided by Dr. M. Peterson of Dow AgroSciences (above address).

[9] SAS statistical software, Version XX, Statistical Analysis Systems Institute, Inc., 100 SAS Campus Drive Cary, NC 27513-2414.

[10] SigmaPlot graphing software, 233 South Wacker Drive, #11, Chicago, IL 60606.

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