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A quick test using seeds for detecting dicamba resistance in fathen (*Chenopodium album*)

Hossein Ghanizadeh[1], Kerry C. Harrington[1*], Trevor K. James[2], David J. Woolley[1]

[1]Institute of Agriculture and Environment, Massey University, PB 11-222, Palmerston North 4442, New Zealand
[2]AgResearch, Ruakura Research Centre, PB 3123, Hamilton 3240, New Zealand

***Corresponding author: K.Harrington@massey.ac.nz**

Abstract

A quick test was developed using seeds in petri dishes to detect resistance to dicamba in fathen (*Chenopodium album* L.) which has developed in some maize fields in Waikato, New Zealand. Seeds were collected from four Waikato maize fields (populations A, B, L and M) where dicamba has been applied for many years, and also three sites (populations C, P and Y) where dicamba was unlikely to have been used. Following a dormancy-breaking procedure, seeds of each population were germinated in petri dishes containing a range of dicamba concentrations from 0.02 to 0.32 mg ae L^{-1}. The length of seedling hypocotyls and radicles was measured 14 days later, and these were found to be significantly shorter at most dicamba concentrations for five of the populations (A, B, C, P and Y) compared with the seedlings from populations L and M at the same concentrations. Dose response curves estimated populations L and M were 22 and 48 times more resistant respectively than the other five populations. The seven populations were also grown in pots in two separate greenhouse experiments, sprayed with a range of dicamba rates after 1 month then shoot weight was measured 7 weeks later. The greenhouse experiments confirmed that populations L and M were the only two dicamba resistant populations. The petri dish test was much quicker and has seldom been used previously for auxinic herbicides but over-estimated the levels of resistance which were approximately 7-fold and 19-fold for populations L and M respectively in the greenhouse experiments.

Keywords: Auxinic herbicide resistance, *Chenopodium album*, dose-response, quick test, seed.
Abbreviations: Ae_ acid equivalent, drc_dose-response curves, GR_{50}_50% growth reduction.

Introduction

The selection pressure from continuous use of herbicides with the same mode of action over many decades for controlling weeds has led to the appearance of herbicide resistant biotypes of weed species (Heap, 1997). Auxinic herbicides (especially 2,4-D and MCPA) are some of the earliest compounds introduced for selective control of broad-leaved weeds in cereals (Sterling and Hall, 1997). Although auxinic herbicides have been applied extensively however, to date only 31 weed species have been discovered resistant to any herbicide from this group (Heap, 2014). Dicamba is an auxinic herbicide which is applied at post-emergence to control annual and perennial broad-leaved weeds in grain crops and pastures (Rahman et al., 2014). Recently, it was reported that *Chenopodium album* (fathen) has evolved resistance to dicamba in New Zealand following many years of application to triazine-resistant fathen in maize crops (James et al., 2005). To our knowledge, this is the first and only case of dicamba-resistant fathen reported so far in the world. To manage cases of herbicide resistance, it is useful to have an easy way of determining which weeds are resistant. Screening for weeds resistant to herbicides usually involves either greenhouse studies in which plants are grown in pots then sprayed (greenhouse dose-response tests) or else laboratory assays, often known as quick tests (Beckie et al., 2000). Although the greenhouse dose-response tests produce results that most closely simulate field conditions, such tests often take many weeks to complete and can occupy large areas of greenhouse space when a large number of populations need to be tested (Burgos et al., 2013). Developing quick tests for detecting herbicide resistant weeds has an important role in managing herbicide resistance as these methods are usually less expensive, can be completed more rapidly and are not space-limiting compared with the greenhouse dose-response tests (Koger et al., 2005). However quick tests ideally should be easy to undertake and must accurately detect resistance (Beckie et al., 2000). Quick tests that have previously been developed for screening weed samples suspected to be resistant to herbicides have included petri dish assays (Cirujeda et al., 2001, Tal et al., 2000), chlorophyll fluorescence (Norsworthy et al., 1998), leaf disk assays (Hensley, 1981, Shaner et al., 2005), pollen germination (Richter and Powles, 1993) and whole plant studies (Boutsalis, 2001). When developing new quick tests, it is important to compare results from such tests with the greenhouse dose-response tests in order to examine the reliability of the quick tests (Beckie et al., 2000).To our knowledge, most quick tests have been developed for detecting resistance to herbicides from groups other than the auxinic herbicides (Burgos et al., 2013). There is almost no published work on quick tests using seeds for determining the presence of resistance to auxinic herbicides. The objectives of this study were to develop a simple, reliable assay for detecting dicamba resistance in fathen using seeds, and to compare the results of this quick test with conventional greenhouse dose-response trials to validate the reliability of this quick test for detecting dicamba resistance in fathen.

Results

Optimum concentration for identifying resistant fathen in petri dish assays

In each of the petri dish assays where fathen seedlings were germinating within dicamba solutions, the herbicide caused inhibition of root elongation, radical swelling and stunted hypocotyl growth when compared to the untreated control seedlings. Different responses were observed consistently between the seven populations in all assays, which showed that two of the populations were more resistant to dicamba than the other populations tested. With increasing dicamba rate, all populations had shorter hypocotyls and radicles compared to their untreated controls (Fig 1a and b). The effect of dicamba on radicle length was far more severe than on hypocotyl length, and even at the lowest dicamba concentration used in this study, a very short radicle was still observed for the seedlings of susceptible populations (Fig 1a and b). Results from Experiments 1 and 2 showed that a dicamba concentration of 0.16 mg ae L^{-1} gave the best differentiation between resistant and susceptible populations (Fig. 1a and b). The radicle length and hypocotyl length relative to the untreated control for five populations (populations A, B, C, P and Y) were significantly less than that of populations L and M at this herbicide concentration, indicating that only populations L and M were resistant to dicamba (Fig. 1a and b). Radicle length and hypocotyl length for resistant populations were almost unaffected at 0.16 mg ae L^{-1} of dicamba compared to susceptible populations. The radicle and hypocotyl length of both populations L and M were significantly greater than those of the other populations at all concentrations of dicamba. The radicle length of population L was significantly lower than that of population M at 0.08 and 0.16 mg ae L^{-1} of dicamba (Fig. 1a). Also, a significant reduction in hypocotyl length was recorded for population L compared to population M at 0.32 g ae ha^{-1} of dicamba (Fig. 1b). These results suggested that population M was more resistant to dicamba than population L.

Estimating magnitude of resistance using petri dish assay

By increasing the concentrations applied to seeds from populations L and M in Experiments 3 and 4, full dose response curves for all of the populations could be produced based on reduction in hypocotyl length (Fig. 2). The three-parameter logistic model provided a good fit to the data ($P<0.0001$; $R^2 \geq 0.98$). The concentration of dicamba which caused 50% reduction in the length of hypocotyl (GR_{50}) differed between populations with the GR_{50} values for populations L and M significantly higher than those for the other populations. The level of resistance to dicamba in populations L and M when compared against the other populations was calculated based on relative GR_{50} values (Table 1). As occurred in Experiments 1 and 2, a greater level of resistance to dicamba for population M was detected than for population L. The apparent level of resistance to dicamba for population L ranged from 22 to 31 times greater than the susceptible populations (A, B, C, P and Y), with the variability due to differences in GR_{50} of the various susceptible populations. The average estimate was a 26-fold level of resistance for population L, compared with a 55-fold level of resistance for population M, with estimates of the level of resistance for population M ranging from 47 to 65 times greater than the susceptible populations.

Validating petri dish results using greenhouse dose-response tests

When the seven fathen populations were evaluated in two greenhouse experiments (Experiments 5 and 6), only populations L and M were found to be resistant to dicamba (Fig. 3a and b), showing that the petri dish assays had correctly detected which populations were resistant. The three-parameter logistic model provided a good fit to the data as indicated by $R^2 \geq 0.96$ and $P<0.0001$. For all populations, each of the plants treated with a rate of dicamba were affected the same as other plants from that population treated with the same rate of herbicide, suggesting that seeds collected for each population were homogenous in their response to dicamba. The shoot fresh weight of plants from all populations except populations L and M was reduced by 50% (compared with untreated plants) at an application rate of approximately 800 g ae ha^{-1} of dicamba, whereas significantly much higher rates were required to cause a 50% reduction in shoot fresh weight for populations L and M (Fig 3a and b). Plants of populations A, B, C, P and Y treated with 800 g ae ha^{-1} of dicamba were severely damaged, whereas for populations L and M, no visual injury was evident at this rate of dicamba. When the ratio of resistance of one population against the other populations was calculated based on GR_{50} (the rate of herbicide which caused 50% reduction in fresh weight) values (Table 2), the levels of resistance to dicamba in population L were estimated to be 13 times (ranging from 11.1 to 15.2 times) and 8 times (range from 5.1 to 10.8 times) greater than the susceptible populations (A, B, C, P and Y) in the first and second greenhouse dose-response tests respectively. In contrast, the levels of resistance in population M were estimated to be 16 times (range of 14.0 to 19.3 times) and 10 times (range of 6.9 to 14.5 times) greater than the susceptible populations in the first and second greenhouse dose-response tests respectively.

Discussion

The petri dish experiments showed that the length of hypocotyls and radicles of fathen seedlings was reduced with exposure to increasing dicamba concentrations. Similar observations have been reported for the seedlings of *Sinapis arvensis* when treated with different solutions of various auxinic herbicides including dicamba (Wei et al., 2000). However, the radicle of susceptible populations was particularly sensitive to dicamba compared with the hypocotyl, giving the impression that the resistant populations were many times more resistant than susceptible populations than what was found in the greenhouse dose-response tests. Similarly, it has been reported that MCPA (an auxinic herbicide) caused a significant reduction in radicle length of the seedlings of *Brassica napus* (Polit et al., 2014). As measurements of hypocotyl length gave good indications of differences in susceptibility to dicamba between the populations tested, further experiments focused on just the hypocotyl rather than decreasing dicamba concentrations sufficiently to get dose response curves for comparing populations using radicle length. The results of this work showed that the petri dish quick test could reliably detect dicamba resistance in fathen. So far, quick tests using seeds have mainly been used for detecting resistance to glyphosate (Perez and Kogan, 2003) and ACCase inhibitor herbicides (Tal et al., 2000). The only assay for resistance to auxinic herbicides that has used seeds found in the literature involved *Papaver rhoeas* resistant to 2,4-D, in which use was made of different responses in the length of seedling shoots of resistant

Table 1. The parameters (see footnote) estimated from the nonlinear regression analysis of hypocotyl growth reduction caused by a range of dicamba concentrations on seedling germination of seven fathen populations (A, B, L and M from Waikato maize fields, C, P and Y from Palmerston North) in Experiments 3 and 4 (data pooled). Each resistant population has been compared against each susceptible population for estimating level of resistance.

Population	d	b	GR_{50}	R/S (A) GR_{50} ratio	R/S (B) GR_{50} ratio	R/S (C) GR_{50} ratio	R/S (P) GR_{50} ratio	R/S (Y) GR_{50} ratio	R^2
A	98.8	1.3	0.063c*	-	-	-	-	-	0.98
B	98.9	1.4	0.059c	-	-	-	-	-	0.99
C	99.2	1.0	0.058c	-	-	-	-	-	0.99
L	102.7	1.3	1.415b	22.4	23.9	24.4	30.7	27.7	0.98
M	99.7	1.0	3.004a	47.7	50.8	51.7	65.2	58.8	0.99
P	99.8	0.9	0.046c	-	-	-	-	-	0.99
Y	99.3	0.9	0.051c	-	-	-	-	-	0.99
P value	0.99	0.50	<0.0001						

d = the upper limit, b = the slope around the GR_{50}, GR_{50} = the concentration of herbicide (mg ae L^{-1}) required to reduce hypocotyl length by 50%, R/S GR_{50}=resistant/susceptible GR_{50} ratio. R^2 = coefficient of determination. * Mean values within each column followed by the same letters are not significantly different at 5% probability according to Tukey's tests

and susceptible populations when exposed to 2,4-D (Torra et al., 2010). However, dose response curves were not calculated in that work to allow an estimate of the difference in resistance between populations. Differences between dose response curves created using the seedling hypocotyl length suggested that population L was about 26 times more resistant than susceptible populations to dicamba, and that population M was about 55 times more resistant. Yet with the greenhouse dose-response tests, the level of dicamba resistance for population L appeared to be between 5-fold to 15-fold, and for population M, it was between 7-fold to 19-fold. During normal spraying of weeds in fields, seedlings are similar in size and growth form to what were used in the greenhouse dose-response tests, so presumably the level of resistance estimated in the greenhouse experiments is similar to what is found in the field (Burgos et al., 2013). The validity of the estimates provided by the greenhouse dose-response tests can be explored by comparing with results that have been obtained in the field. Severe injuries were recorded for populations A, B, C, P and Y at 800 g ae ha^{-1} (recommended rate) compared to populations L and M that had no visual injury at this rate, which suggested that dicamba resistance had evolved for populations L and M. The visual injuries recorded for populations A, B, C, P and Y as dicamba susceptible populations were similar to those reported previously by James et al. (2005) for susceptible plants. Populations L and M were collected from maize fields near to the area where dicamba resistance has been reported previously (Rahman et al., 2014). In a study conducted in two of these nearby maize fields, dicamba-resistant fathen populations were found to survive a dicamba rate eight times higher than the recommended field rate (Rahman et al., 2008). In another published case of dicamba resistance, a level of resistance up to 30-fold has been reported for dicamba resistant *Kochia scoparia* (Preston et al., 2009). So although the petri dish test could reliably detect which populations were resistant to dicamba, the magnitude of resistance to dicamba was over-estimated. Other workers who have also developed quick tests using seeds for detecting resistance to herbicides from different modes of action have also found that the quick tests tend to over-estimate the extent of resistance. For example, Burke et al. (2006) reported that the level of resistance to clethodim and fluazifop in *Sorghum halepense* was over-estimated in their petri dish assay compared with greenhouse dose-response trials. Despite over-estimating the magnitude of resistance, the petri dish assay remains a useful test for detecting resistance to dicamba

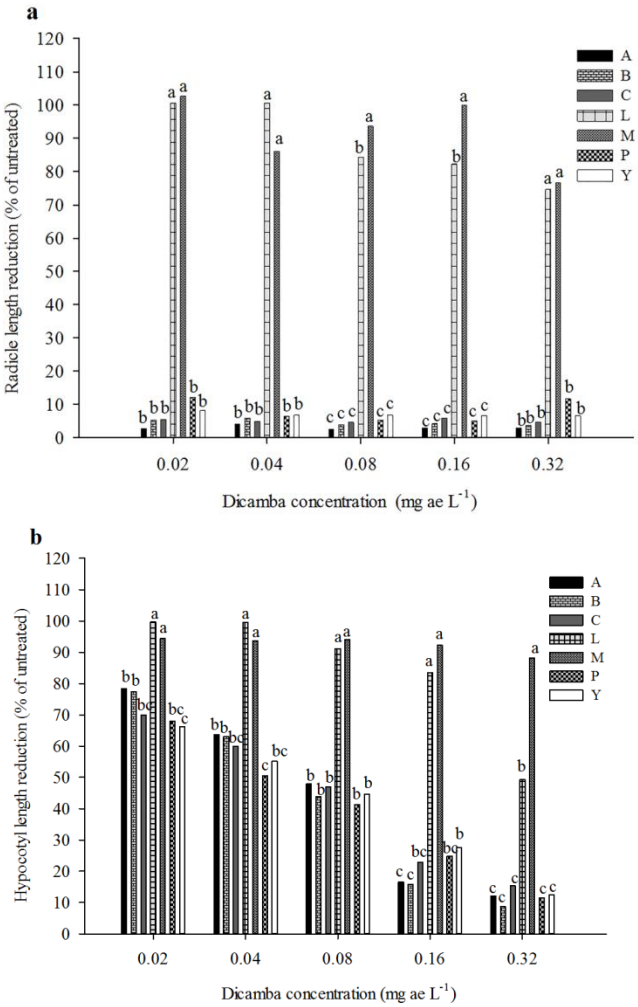

Fig 1. Effect of different dicamba concentrations on (a) radicle length and (b) hypocotyl length of germinating seedlings for seven populations of fathen (A, B, L and M from Waikato maize fields, C, P and Y from Palmerston North waste areas) relative to untreated control in Experiments 1 and 2 (data pooled). Mean values within each concentration of dicamba with the same letters were not significantly different at 5% probability according to Fisher's (LSD) tests.

Table 2. The parameters (see footnote) estimated from the nonlinear regression analysis of shoot fresh weight reduction of potted plants from seven fathen populations (A, B, L and M from Waikato maize fields, C, P and Y from Palmerston North) sprayed with a range of dicamba rates in Experiments 5 and 6. Each resistant population has been compared against each susceptible population for estimating level of resistance.

Population	d	b	GR50	R/S (A) GR_{50} ratio	R/S (B) GR_{50} ratio	R/S (C) GR_{50} ratio	R/S (P) GR_{50} ratio	R/S (Y) GR_{50} ratio	R^2
Experiment 5									
A	95.7	1.3	619c*	-	-	-	-	-	0.96
B	94.8	2.4	706c	-	-	-	-	-	0.96
C	94.3	2.9	816c	-	-	-	-	-	0.98
L	96.1	1.5	9438b	15.2	13.4	11.6	11.6	11.1	0.97
M	96.5	3.2	11979a	19.3	16.9	14.7	14.7	14.0	0.98
P	97.7	2.1	814c	-	-	-	-	-	0.98
Y	94.9	3.6	853c	-	-	-	-	-	0.97
P value	0.96	0.11	<0.0001						
Experiment 6									
A	100.7	0.8	664c	-	-	-	-	-	0.98
B	99.7	0.8	496c	-	-	-	-	-	0.99
C	101.1	1.0	816c	-	-	-	-	-	0.96
L	99.9	1.1	5335b	8.0	10.8	6.5	5.1	7.5	0.98
M	98.4	1.1	7177a	10.8	14.5	8.8	6.9	10.0	0.96
P	99.4	1.1	1047c	-	-	-	-	-	0.99
Y	99.3	1.3	711c	-	-	-	-	-	0.97
P value	0.99	0.80	<0.0001						

d = the upper limit, b = the slope around the GR_{50}, GR_{50} = the rate of herbicide (g ae ha^{-1}) required to reduce fresh weight by 50%, R/S GR_{50}=resistant/susceptible GR_{50} ratio. R^2 = coefficient of determination. * In each experiment, mean values within each column followed by the same letters are not different at 5% probability according to Tukey's tests.

within fathen populations as this test meets the features of a good quick test for screening resistant weeds (Beckie et al., 2000). By placing fathen seeds in petri dishes containing 0.16 mg ae L^{-1} of dicamba, presence or absence of resistance can be determined within 2 weeks. In contrast, the greenhouse dose-response test would take about four times longer and need sufficient space in a greenhouse for the plants to grow properly. The quick test needs only petri dishes and perhaps space in a seed germination cabinet, though it could probably be conducted on a window-sill. Therefore, this test enables easy testing of a large number of fathen populations to determine whether dicamba resistance has evolved. Currently, it is not known how widespread the dicamba resistant fathen is within New Zealand. Such information is useful for management of the problem and can be obtained in a short period using the petri dish assay developed in this study. By collecting seed from fathen plants within maize fields near harvest time, it would be possible to determine if they are present because they missed being sprayed by dicamba or whether resistance is developing. To our knowledge, this is the first use of an assay using seeds for detecting dicamba resistance. Further investigations are needed to study if this petri dish assay could be adapted for detecting other auxinic herbicide resistance, such as for the nodding thistle (*Carduus nutans*) (Harrington and Woolley, 2006) and giant buttercup (*Ranunculus acris*) populations that have developed resistance to MCPA in parts of New Zealand (Bourdôt et al., 1990). The result of the greenhouse dose-response tests also showed that population M was more resistant to dicamba than population L and this was in agreement with the results of the petri dish assays. Thus the petri dish assay could also be used to screen for differences in levels of resistance amongst resistant populations, even if it is not suitable to correctly quantify the precise level of resistance. The petri dish assay could be used as in Experiments 3 and 4 to develop dose response curves, or more simply by testing populations found to be resistant

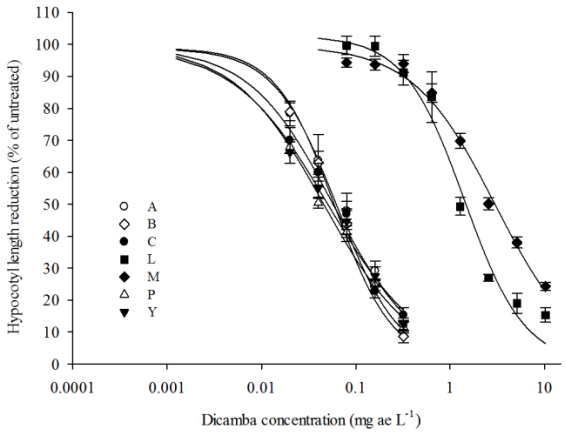

Fig 2. Fitted dose response curves (on logarithmic dose scale) for seven populations of fathen (A, B, L and M from Waikato maize fields, C, P and Y from Palmerston North) for effect of dicamba on the length of seedling hypocotyls in Experiments 3 and 4 (data pooled). Vertical bars represent ± standard error of the mean.

using a concentration such as 0.32 mg ae L^{-1} which separated out populations L and M in Experiments 1 and 2. Further research is needed to determine why differential levels of dicamba resistance were recorded between populations L and M in this study.

Materials and Methods

Plant materials

All experiments were conducted using seed collected in New Zealand from fathen populations growing either in Waikato maize fields near Matamata with long histories of herbicide

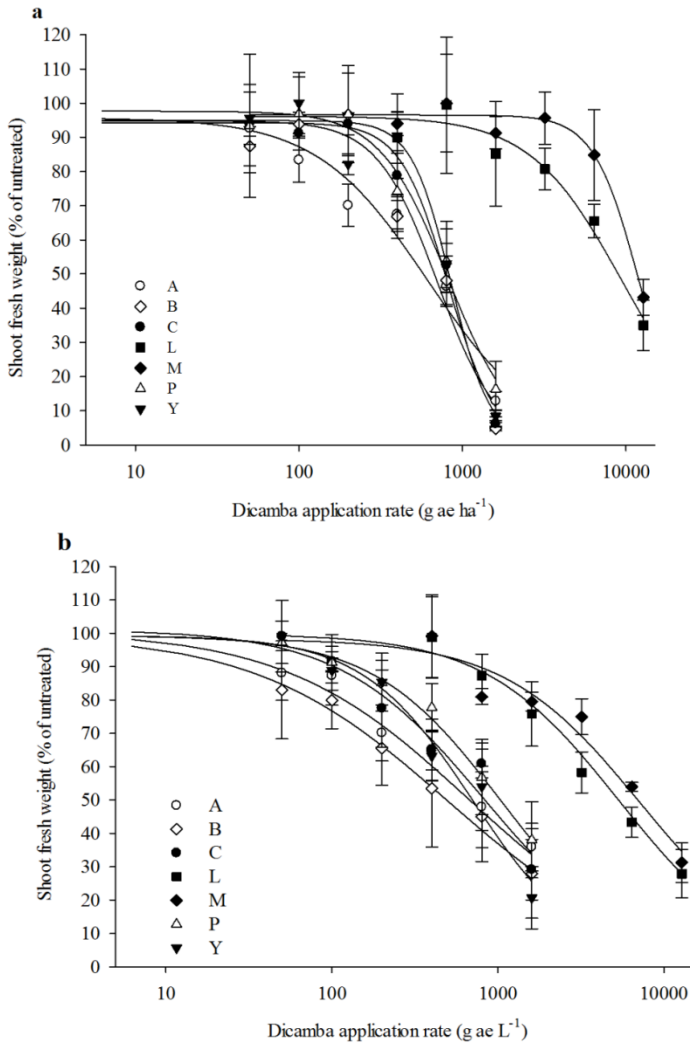

Fig 3. Fitted dose response curves (on logarithmic dose scale) of the reduction in shoot fresh weight for seven populations of fathen (A, B, L and M from Waikato maize fields, C, P and Y from Palmerston North) following application of dicamba to potted plants in Experiments (a) 5 and (b) 6. Vertical bars represent ± standard error of the mean.

use (populations A, B, L and M) or from Palmerston North waste areas thought not to have been sprayed with no herbicides in recent years (populations C, P and Y). The maize fields from which seeds of populations L and M were collected were known to contain populations of fathen that were no longer susceptible to dicamba due to persistent use of this herbicide over the previous ten years.

Initial petri dish experiments

Techniques were developed initially to ensure dormancy of the fathen seeds could be broken so that seeds would germinate uniformly in all further experiments. After trying several techniques, the best method was found to be pre-treating seeds for 7 days in potassium nitrate in the dark at 5°C following removal of perianths (data not shown). So for all petri dish experiments, seeds were put on Steel Blue Seed Germination Blotters (Anchor Paper, Minnesota, USA) (hereinafter referred to as blotters) saturated with 0.02% KNO_3 (potassium nitrate) after the perianths had been removed, with 20 seeds used per replicate of each treatment. The blotters were placed in petri dishes which were kept for 7 days at 5°C in the dark. In Experiment 1, after this 7 day dormancy breaking period, all populations were treated with solutions of dicamba (Kamba 500, dimethylamine salt) at concentrations of 0.02, 0.04, 0.08, 0.16 and 0.32 mg ae L^{-1}. After the herbicide solutions were added to the petri dishes containing seeds, the lids of the dishes were sealed with cling wrap in order to decrease evaporation losses before the dishes were put into a growth chamber under constant light at 25°C. Light was provided by four 40 W fluorescent white tubes giving a photon flux density of 30 µmol m^{-2} s^{-1}. A randomized experimental design was used with three replicates. An untreated control was included for each population and each replicate consisted of a petri dish of 20 seeds. This experiment was then repeated (Experiment 2).

Petri dish dose-response tests

In Experiment 3, the same procedures were used as described above for Experiments 1 and 2, except a different set of herbicide concentrations was used for populations L and M which had been found to be more resistant to dicamba than the other five susceptible populations (A, B, C, P and Y). These five susceptible populations were treated with the same concentrations used in Experiments 1 and 2, whereas populations L and M were treated with 0.08, 0.16, 0.32, 0.64, 1.28, 2.56, 5.12 and 10.24 mg ae L^{-1} of dicamba. The experiment was then repeated (Experiment 4).

Measurements for petri dish assays

The main effect of the dicamba on seedling development was found to be stunting of growth for both the radicles and hypocotyls. In Experiments 1-4, after 14 days, the average radicle length and hypocotyl length of germinated seeds was determined using a calibrated scale printed on to an acetate sheet aided by a dissecting microscope. The reduction in length of treated radicles and hypocotyls relative to those from the untreated control seedlings was then calculated for each herbicide rate.

Greenhouse dose-response tests

Greenhouse experiments (Experiments 5 and 6) were conducted in order to validate the results of the petri dish assays. In Experiment 5, plants were grown using seeds from the same populations used in the petri dish assays. Five seeds were planted in each pot, which were polythene planter bags containing 450 ml of bark-based potting mix, and these were placed in a temperature-controlled greenhouse on 20 December 2013 with automated capillary irrigation. Over 80% of the seeds had germinated after 2 weeks for all populations, and they were thinned to one plant per pot when seedlings were at the 3-4 leaf stage. When plants were 13-14 cm tall, several rates of dicamba (same formulation as for the seed-based assay) were applied on 25 January 2014 using a laboratory track sprayer calibrated to deliver 227 L ha^{-1} at 200 kPa. Susceptible populations (A, B, C, P and Y) received 0, 50, 100, 200, 400, 800 and 1600 g ae ha^{-1} of dicamba. Resistant populations (L and M) were treated with 0, 400, 800, 1600, 3200, 6400 and 12800 g ae ha^{-1} of dicamba. A randomised complete block design was used with five replicates (one plant for each replicate). The average temperature in the 2 weeks after spraying was 20.1 °C and all above-ground plant tissue was harvested 7 weeks after

spraying and weighed. This experiment was then repeated (Experiment 6), with seeds sown on 10 April 2014, sprayed on 16 May 2014 (plants were 15-16 cm tall) and the average temperature in the 2 weeks after spraying was 17.5 °C. Daylight was supplemented using two 500 W hydrogen gas lamps to maintain a 14 h day-length to try stopping the plants from flowering immediately.

Statistical analysis

For each pair of identical petri dish assays, a two-way analysis of variance compared the results from each assay and no significant interaction was found between data for seed-based assays ($P>0.05$), so the data were pooled. The data from the greenhouse dose-response studies were not pooled. The pooled data of the Experiments 1 and 2 were examined using a one-way ANOVA using SAS v. 9.4 and means were separated using Fisher's (LSD) test at a 5% level of probability. A three-parameter logistic model was fitted to the pooled data from petri dish assays (Experiments 3 and 4) and the data of the greenhouse dose-response studies from Experiments 5 and 6 separately, using the following equation:

$$Y = [d/(1 + \exp(b(\log(x) - \log(GR_{50}))))]$$

where Y was hypocotyl length or plant biomass as a percentage of control, d was the upper limit, x was herbicide rate, GR_{50} was the rate of herbicide corresponding 50% reduction in hypocotyl length or plant biomass and b was the slope around the GR_{50}. The data were fitted to this model using the statistical software R (Version 2.15.2) with its dose-response curve (drc) package (Knezevic et al., 2007). A one-way ANOVA was performed to compare parameters estimated from the dose-response tests for the fathen populations using Graphpad Prism v.5 and means were separated using Tukey's test at a 5% level of probability (Ademola and Eloff, 2011).

Conclusion

The presence of fathen plants in a field that have developed resistance to dicamba can be reliably detected by collecting seeds from these plants and conducting a quick test which involves germinating the seeds in a petri dish on blotters soaked with a dicamba solution. The simplest form of this test would be to use just one concentration, probably 0.16 mg ae L^{-1} of dicamba, to separate resistant from susceptible plants, comparing their growth with seedlings germinating in the absence of dicamba. Seeds could be collected from plants noticed by farmers to have survived dicamba applications in a maize field and these could then be screened with the quick test to determine if these plants merely missed being sprayed or are the first plants in a field to develop resistance. If resistance is detected, farmers could be warned to start using alternative herbicides to prevent the resistance from developing further.

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