Research Article

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Development of a rapid detection assay for acetolactate synthase inhibitors resistance in three *Amaranthus* weed species through loop-mediated isothermal amplification

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Abstract

BACKGROUND: The early detection of herbicide resistance in weeds is a key factor to avoid herbicide waste and improve agriculture sustainability. The present study aimed to develop and validate an allele-specific loop-mediated isothermal amplification (AS-LAMP) assay for the quick on-site detection of the resistance-endowing point mutation Trp-574-Leu in the acetolactate synthase (*ALS*) gene in three widely diffused *Amaranthus* weed species: *Amaranthus retroflexus*, *Amaranthus hybridus* and *Amaranthus tuberculatus*.

RESULTS: The AS-LAMP protocol was developed on wild-type and *ALS*-mutant plants of the three species and revealed that the amplification approach with only the primer set specific for the mutant allele (574-Leu) was the most promising. The validation and estimation of the AS-LAMP performance evaluated by comparing the results with those of the molecular marker (cleaved amplified polymorphic sequences) indicated that, although the sensitivity and specificity were relatively high in all species (overall 100 and > 65%, respectively), precision was high for *A. hybridus* L. and *A. retroflexus* L. (75 and 79%, respectively), but quite low for *A. tuberculatus* (Moq.) J. D. Sauer (59%). The LAMP assay was also effective on crude genomic DNA extraction, allowing the quick detection of mutant plants in field situation (on site resistance detection).

CONCLUSION: The proposed AS-LAMP method has proven to be a promising technique for rapid detection of resistance as a result of Trp-574-Leu on the two monoecious weedy *Amaranthus* species but resulted less effective in the genetically variable dioecious species *A. tuberculatus*.

Keywords: ALS inhibitors; detection; herbicide resistance; LAMP; pigweed; waterhemp

INTRODUCTION

The rapid spread of herbicide resistance is of concern for farmers worldwide because the resulting effectiveness of herbicides is a threat to both crop yield and sustainability. To date, resistance to five or more herbicide sites of action (SoAs) has been reported for 15 weed species and four of them belong to the *Amaranthus* genus: *Amaranthus palmeri* S. Watson, *Amaranthus tuberculatus* (Moq.) J. D. Sauer, *Amaranthus hybridus* L. and *Amaranthus retroflexus* L.[1] The most common resistance evolved by amaranths is against the inhibitors of the acetolactate synthase (ALS) and is mostly a result of mutations in the *ALS* gene, normally present in a single copy,[2] because amaranths are diploid organisms.[3] The first ALS-resistant amaranth in Italy was reported in 2007, and the spread of resistance was relatively slow until 2020 when a more complex situation was reported, with the presence of three ALS-resistant amaranth species (*Amaranthus tuberculatus*, *Amaranthus retroflexus* and *A. hybridus*).[4] More recently, ALS-resistant *A. palmeri* has also been added to this list.[5] The early and rapid detection of resistant weeds is critical to prevent further resistance selection and mitigate their impacts on agriculture.[6] Knowing resistance-endowing traits within a weed population allows the adoption of integrated weed management more appropriate and effective. Currently, to determine whether a suspect population is resistant or not to a specific herbicide, seeds are collected at weed maturity and are then used for dose–response whole-plant herbicide bioassays. Whole-plant assays are still

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preferred over other methods for detecting herbicide resistance,[7] although they are very laborious, plus the response is normally applicable to weed management in the following growing season only. Instead, for situations where the alteration of DNA sequence or structure is known to be the main resistance mechanism, a plethora of in-season DNA-based methods does exist, that are basically variants of PCR.[8] They include restriction fragment length polymorphism, PCR amplification of specific alleles, cleaved amplified polymorphic sequences (CAPS), derived CAPS, real-time PCR and next-generation sequencing.[9] Very recently, also droplet digital PCR[10] and third generation long-read sequencing technologies (e.g. Pacific Biosciences Menlo Park, CA, USA; and Oxford Nanopore Technologies, Oxford, UK) have been applied to detect herbicide resistance.[11] Despite those latter methods are faster than whole-plant assays (days *versus* weeks), they still require a very clean and settled location, expensive chemicals, energy-intensive thermal cyclers and specific data analysis, and therefore are not suitable for rapid on-site resistance detection.

Loop-mediated isothermal amplification (LAMP) assay is a relatively novel molecular method which amplifies target nucleic acids under isothermal conditions (i.e. a constant temperature ranging from 60 to 65 °C) as a result of the use of non-standard strand-displacing polymerases. Four to six primers are specifically designed to amplify a short target sequence, usually 300 bp, allowing the formation of hairpin DNA structures and ensuring high specificity. Depending on the developed protocol and the equipment, results can be read at endpoints (colorimetric assays) or in real-time. The assay gives results in less than 1 h and may be carried out through portable instruments making the technique a very promising DNA-based method suitable for on-site detection of target site resistance. To detect point mutations, primers are designed to recognize specific alleles, and the assay is called allele specific (AS) LAMP.[12] Although there are many examples of LAMP assays to detect resistance to fungicides, antibiotics and insecticides,[13-15] very studies exist on detecting herbicides resistance in weeds, and all examples refer to monocots. Two studies focused on the detection of acetyl-CoA carboxylase mutations: one at position Ile-1781-Leu in *Beckmannia syzigachne* Steud.[16] and one at position Ile-2041-Asn in *Alopecurus aequalis* Sobol.[17] Despite the resistance to ALS-inhibitors involves worldwide the highest number of species (172, up to now),[1] and resistance is mainly a result of point mutations (at least in dicots), to date, only two studies have described LAMP protocols to detect *ALS* mutations, and they are both in *Lolium* spp.[18,19]

The present study aimed to develop an AS-LAMP assay to rapidly detect the resistance-endowing point mutation Trp-574-Leu in the acetolactate synthase (*ALS*) gene in the most common *Amaranthus* species in Italy, *A. tuberculatus*, *A. retroflexus* and *A. hybridus*. A CAPS assay, previously developed,[20] was used as reference method to detect the presence of the 574-Leu *ALS* allele. An on-site protocol using a fast DNA extraction kit was also tested to possibly help the stakeholders to prompt the detection of resistance as a result of this point mutation and take timely actions to limit their spread.

MATERIALS AND METHODS

Plant material

Twelve populations, four for each *Amaranthus* species (*A. hybridus*, *A. retroflexus* and *A. tuberculatus*) were used for the setup and the validation of the AS-LAMP. An additional *A. hybridus* population was added for the simulated field detection step. Three ALS-susceptible checks, one per species (Tu-1, Re-1 and Hy-1), and an ALS-resistant population of *A. tuberculatus* (Tu-3) were available from previous studies.[4] Other putatively ALS-resistant populations (Tu-4, Re-3, Re-4, Hy-3 and Hy-4) were collected from ALS-treated soybean fields, while putatively ALS-susceptible populations (Tu-2, Re-2 and Hy-2) were collected from never-treated areas. Mature seeds were collected from at least 25–30 plants. The additional putatively resistant *A. hybridus* population was collected from a rice field (Hy-5).

Assessment of resistance to ALS-inhibiting herbicides

To assess the resistance status of the newly collected populations, plants were treated with the field doses of imazamox and thifensulfuron-methyl, two ALS inhibitors. Plant growth and herbicide treatments were carried out as previously described.[4] The bioassay was performed twice in a greenhouse located in north-eastern Italy (45°21′N, 11°58′E) where temperatures ranged from 15 to 20 °C and from 25 to 34 °C, during night and day, respectively. Seeds were sown in 0.6% agarose medium, vernalized at 4 °C for 7 days, germinated at 28 °C for 12 h/18 °C for 12 h in a cabinet for 5 days, and then transplanted in standard potting mix (20 seedlings per pot/replicate, with two replicates per each herbicide dose). At three- to four- leaf stage (13–14 of the BBCH scale),[21] plants were treated with thifensulfuron-methyl at 6 g a.i. ha^{-1} (Harmony 50 SX; FMC Agro Italia, Bergamo, Italy; 500 g a.i. kg^{-1}) and imazamox at 40 g a.i. ha^{-1} (Tuareg®; Corteva, Cremona, Italy; 40 g a.i. L^{-1}) and at three-times that doses. The herbicides were applied using a precision bench sprayer delivering 300 L ha^{-1} at a pressure of 215 kPa and speed of approximately 0.75 m s^{-1}, with a boom equipped with three flat-fan (extended range) hydraulic nozzles (model 11002; Teejet, Glendale Heights, ILUSA). Four weeks after herbicide application, the number of surviving plants was assessed and expressed as a percentage with respect to the untreated plants.

DNA extraction

For the molecular analyses, approximately 1 cm^2 of leaf per plant was sampled and stored at −20 °C until the genomic DNA extraction was carried out using a cetyltrimethylammonium bromide (CTAB) protocol adapted for room-temperature handling.[5,22] Briefly, thawed samples were grinded with tissue TissueLyser II (Qiagen, Hilden, Germany) at room temperature for 1 min at 30 Hz; the ground tissue was incubated with 600 µL of 2% CTAB plus 1% polyvinylpyrrolidone buffer (without mercaptoethanol) at 60 °C for 30 min; 600 µL of 24:1 chloroform: isoamyl alcohol was added after incubation and gently mixed by inversion; samples were centrifuged for 20 min at 10 000 × *g*; the aqueous phase was recovered and DNA precipitated with 200 µL of cold (−20 °C) isopropyl alcohol and centrifuged for 20 min at 10 000 × *g*; all liquid was removed, and the pellets were rinsed with 70% ethanol and allowed to dry at room temperature; dried pellets were dissolved in water. DNA concentration and quality were determined using the NanoDrop 2000 Spectrophotometer (Thermo Fisher Scientific, Waltham, USA). DNA concentration was adjusted to 20 ng μL^{-1}.

Detection of mutant ALS by CAPS assay

A CAPS assay was used as reference method to genotype plants. Following a previously described protocol,[20] amplicons of 1.394 bp length, including the 574 position, were obtained by using primers Caps_F1 (5′-GGGAAGAATAAGCAACCTCATGTG-3′)

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and 3UTR_R1 (5′-TGGCTGATGAAAGGCAACAC-3′). PCR were performed using GoTaq® G2 Hot Start Polymerase (Promega, Madison, WI, USA) in 15 μL of mixture, including 3 μL of 5× Green GoTaq Flexi Buffer, dNTPs mix (0.2 mM), $MgCl_2$ (0.9 μM), forward and reverse primers (0.2 μM each), 0.075 μL of GoTaq DNA Polymerase, and 1 μL of DNA. Amplification conditions comprised: 95 °C for 2 min; 35 cycles 95 °C for 30 s, 58 °C for 30 s, 72 °C for 1 min 30 s and 72 °C for 5 min. Next, 5 μL of unpurified PCR product was incubated at 37 °C for 1 h with 0.1 μL of the restriction enzyme MunI (*MfeI*) (10 U μL^{-1}) (Thermo Fisher Scientific), 9 μL of water and 1 μL of 10X buffer G. Digested samples were run on a 1% agarose gel: uncut samples were ascribed as homozygous wild-type (WT), whereas completely digested samples (two bands of approximately 514 and 880 bp, respectively) were ascribed as homozygous mutants, and partially digested samples (three bands of 1.394, 880 and 514 pb, respectively) as Leu/Trp-574 heterozygous mutants.

LAMP setup and validation

Primer design

Six LAMP primers (Table 1) (external primers F3 and B3, internal primers FIP and BIP, and loop primers LF and LB) were designed with Primer Explorer (http://primerexplorer.jp/e) using an *ALS* sequence of *A. tuberculatus* as template, as retrieved from a previous study.[23]

Default parameters were used, with minor changes introduced to obtain a higher number of primer combinations (i.e. 'parameter condition' = normal; Tm F1c/B1c = 60–66 °C; Tm F2/B2 = 59–61 °C; Tm F3/B3 = 59–61 °C; GC rate = 60–80%; distances F2–B2 = 120–250 bp; distances loop F1c-F2 = 40–100 bp). Being the two FIP primers specific for different allelic variants (574-Trp and Leu), this LAMP protocol is defined as allele-specific LAMP (AS-LAMP).[12] The two FIP primers differed for the last nucleotide at the 3′ end only (Table 1).

Reaction settings

Real-time LAMP assays were conducted on a Genie II instrument (OptiGene, Horsham, UK) in 10-μL reaction mixtures, containing 6 μL of GspSSD Isothermal Mastermix (ISO-001) (OptiGene), 200 nM F3 and B3 primers, 2 μM FIP and BIP primers and 1 μM each loop primer (FL and BL). Reactions were carried out at 65 °C for 30 min, with no pre-heating. Each sample was separately amplified with a primer mix including FIP3_Trp or FIP3_Leu, to specifically detect the WT 574 allele (Trp) or the mutant allele (Leu).

Table 1. Primers used for the LAMP assay

Primer name	Primer sequence (5′- to 3′)
F3	TGCTCTTGAACAATCAACAT
B3	AATTGCAGCCCTTAAATCG
FIP3_Trp	AAGGATTCCCGAGGTATGTATGTG-TTAGGTATGGTTGTTCAATG
FIP3_Leu	AAGGATTCCCGAGGTATGTATGTG-TTAGGTATGGTTGTTCAATT
BIP	TTCAGAAATCTTCCCGGATATGC-CTCACCTTGGTAACACGG
LF	CCGGTTAGCTTTGTAAAATCGATCT
LB	TTTGCTGAAGCATGTGATATACCAG

Note: Primer FIP3_Trp was added to the WT-specific LAMP mix, whereas FIP3_Leu was used in the mutant-specific LAMP mix. All the other primers were common to both LAMP mixes.
Abbreviation: LAMP, loop-mediated isothermal amplification.

The isothermal master mix contained a fluorescent double-stranded DNA binding dye for the real-time detection of the results. The specificity of the amplification products was confirmed by checking the melting curves obtained with a slow annealing step (0.05 °C s^{-1}, from 95 to 80 °C) with fluorescence monitoring.

Setup

For the setup, WT and mutant plants genotyped with the CAPS assay were analyzed by LAMP. DNA was extracted as described above from 14 plants of a susceptible and a resistant population per each *Amaranthus* species (Table 2). The plants of the resistant populations were chosen among the survivors to the imazamox treatment while plants of susceptible populations were sampled from the non-treated pots of the experiments described in paragraph (Assessment of resistance to ALS-inhibiting herbicides). Each sample was amplified with both the Trp and the Leu-specific primer sets in separate reactions. Because the OptiGene LAMP machine allows the real-time visualization of each amplification curve, it is possible to distinguish from highly specific amplifications from less specific (i.e. delayed) amplifications. Taking advantage of this feature, the time at which the amplification curves reached the 50% of the maximum fluorescence, defined as t_{50}, was used as a measure of specificity, as suggested in a previous study.[18] If a sample resulted amplified by both the reaction mixtures and the difference between the two t_{50} values was lower than a fixed threshold, the sample was considered 'not determined' and excluded from the analyses. Instead, if the difference between the two t_{50} values was higher than the threshold, the delayed amplification was ignored. The threshold was defined after the setup.

Validation

In the validation step, a different set of WT and resistant plants was used. DNA was extracted from 14 plants of a susceptible and a resistant population per each *Amaranthus* species (Table 2). The plants of both the ALS-susceptible and resistant populations were sampled from the non-treated pots of the experiments described in paragraph (Assessment of resistance to ALS-inhibiting herbicides). The presence/absence of the mutant allele was firstly inferred by using the LAMP assay (= predicted condition), then results were compared with those obtained by the reference method; namely, the CAPS assay (= actual condition) (different researchers carried out the LAMP and the CAPS assays). As in the setup, each sample was amplified with both the Trp and the Leu-specific primer sets in separate reactions. The results were used to draw the confusion matrix after classifying each sample as true positive (TP), false positive (FP), true negative (TN), or false negative (FN). Samples were classified as true positive (TP) if resulting in carrying the mutation by both LAMP and CAPS, whereas the false positive (FP) status was assigned to samples resulting in carrying the mutation for the LAMP, but WT for the CAPS. On the other hand, samples were classified as true negative (TN) if resulting in WT by both methods, whereas the false negative (FN) status was assigned to samples resulting in WT for the LAMP, but as carrying the mutation for the CAPS. Two approaches were evaluated: (i) considering both the WT-specific and the mutant-specific LAMP reactions and (ii) considering only the mutant-specific LAMP reaction. TP, FP, TN and FN were used to determine sensitivity (i.e. true positive rate, TPR = TP/P), specificity (true negative rate, TNR = TN/N) and precision (i.e. positive predictive value, PPV = TP/(TP + FP).

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Table 2. Code and sampling site of the *Amaranthus* populations used in the experiments

Species	Experiment	Population code	Sampling site
Amaranthus tuberculatus	Setup	Tu-1[a]	Floodplain
		Tu-2	Floodplain
	Validation	Tu-3[a]	Soybean
		Tu-4	Soybean
Amaranthus retroflexus	Setup	Re-1[a]	City park
		Re-2	Country roadside
	Validation	Re-3	Soybean
		Re-4	Soybean
Amaranthus hybridus	Setup	Hy-1[a]	City park
		Hy-2	Country roadside
	Validation	Hy-3	Soybean
		Hy-4	Soybean
Amaranthus hybridus	Field simulation	Hy-5	Rice

[a] Populations tested for resistance in previous experiments are reported here with different codes.[2]

For both approaches, all the indices were estimated to determine the most suitable approach to be used in the field.

Resistance prediction under simulated field conditions

Since this LAMP assay is designed to help farmers, technicians, and other stakeholders to understand whether a given amaranth population is susceptible to ALS inhibitors before the herbicide treatment, the assay was validated also under simulated field conditions. Plants of a never-tested field population, suspected to be ALS-resistant, were grown as described in paragraph (Assessment of resistance to ALS-inhibiting herbicides) until the two to four leaves stage. Crude (unpurified) genomic DNA samples were prepared with the Plant Material Lysis Kit (EXT-001) (OptiGene), following the producer's protocol, but extending the manual grinding from 1 to 3 min. The crude DNA samples (30 plants) were then amplified with the LAMP assay as described in paragraph (Reaction settings) but using the mutant-specific LAMP primer set only and adapting the reaction to a final volume of 25 µL. The percentage of plants carrying the point mutation was used to infer the resistance status of the population. An herbicide bioassay, carried out as descried in paragraph (Assessment of resistance to ALS-inhibiting herbicides), to determine the effective resistant status of the population, was then used to check the result of the LAMP assay.

RESULTS

Assessment of resistance

Susceptible check populations belonging to the three species (Tu-1, Re-1 and Hy-1) were completely controlled by both herbicides at both doses, indicating that the treatments were effective. Also, populations Tu-2, Re-2 and Hy-2 were completely controlled, resulting in being susceptible to both the ALS-inhibitors, as expected, because they were collected in herbicide-untreated areas. Instead, populations Tu-3, Tu-4, Re-3, Re-4, Hy-3 and Hy-4, all collected in crop fields, had survival rates to imazamox ranging from 81% to 100% and to thifensulfuron-methyl ranging from 79% to 97%, thus resulting in being cross-resistant to ALS inhibitors. No differences were found in the survival rate to the field dose and three times that; thus, for conciseness, only the response to the field rate is discussed (Fig. 1).

LAMP setup

The CAPS assay revealed that all the plants of the ALS-susceptible populations tested (Hy-1, Re-1 and Tu-1) had only the WT allele 574-Trp, such that the amplicons remain uncut after reaction with the restriction enzyme *Mfe*I (e.g. Fig. 2, gel pictures on the top left for pop Hy-1). The CAPS assay conducted on the plants of the ALS-resistant populations had only plants with the mutant allele 574-Leu, such that amplicons are cut by *Mfe*I (e.g. Fig. 2, gel pictures on the top right). Because no undigested amplicons were visible on agarose gel, all the resistant plants were homozygous resistant. Seven out of fourteen of those samples were analyzed by LAMP together with a no-template (blank) control: most samples of the susceptible populations were successfully amplified by the Trp-specific LAMP mixture, but not by the Leu-specific mixture (e.g. Fig. 2, graph on the bottom left). By contrast, most samples of the ALS-resistant populations were correctly amplified by the Leu-specific LAMP mixture, but not by the Trp-specific mixture (e.g. Fig. 2, graph on the bottom right). Some plants, despite being homozygous (WT 574-Trp or mutant 574-Leu), were amplified with both mixtures, indicating that sometimes non-specific amplifications can occur. However, when there is a delay between the specific amplification curve and the non-specific amplification curve equal to or higher than 5 min, it is possible to discriminate both curves and therefore the result is reliable. The discriminating threshold of 5 min was chosen as a result of being detectable even with the naked eye, and it was also suggested previously.[18] As an example, the mutant allele curve of plant number 2 of population Hy-3 ('R2', red lines in Fig. 2, graph on the bottom right) reached 50% of maximum fluorescence at t_{50mut} = 18 min, whereas the WT allele curve reached 50% maximum fluorescence at t_{50wt} = 26 min. The delay between t_{50wt} and t_{50mut} was 8 min and the sample was considered as carrying the mutation. Instead, sample S1 of population Hy-1 (black lines in Fig. 2, graph on the bottom left) was considered as not determined ($t_{50mut} - t_{50wt}$ = 2 min). To optimize the specificity of the LAMP assay, different parameters were considered and evaluated. Different reaction volumes and template DNA concentrations were tested as well as different primer design strategies (FIP primers mutated at the 5′ end, plus the 5′ and 3′ mutated BIP primers) (data not shown). After being optimized, the best conditions

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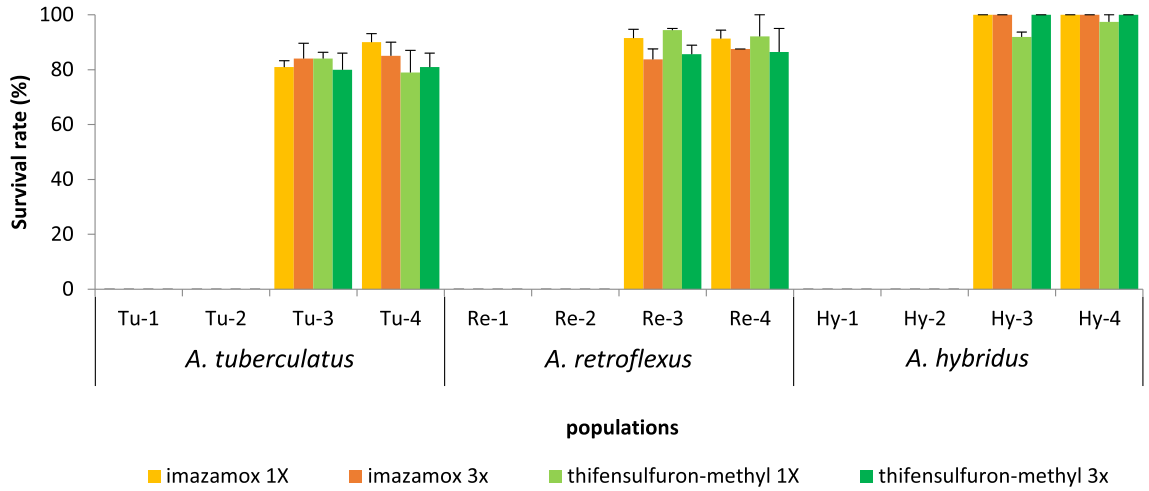

Figure 1. Response to imazamox and thifensulfuron-methyl applied at the field dose (1×) and three-times that (3×). Bars represent the mean percentage of surviving plants and the whiskers represent the standard errors.

described in paragraph (Reaction settings) were adopted for the subsequent experiments (validation).

LAMP validation

After the setup, a different set of plants were used for the validation. Each sample was amplified with the mix specific for the 574-Leu mutant allele and with that specific for the 574-Trp WT allele. The LAMP results were compared with those obtained with the CAPS assay, and each sample was classified as TP, TN, FP or FN to estimate the corresponding common performance indices. The obtained results were firstly analyzed by considering both amplifications and then with only the LAMP specific for the mutant allele, aiming to determine which approach was the most suitable to be applied in a field condition. An example of the interpretation of the results with the two alternative approaches is reported in Figs 3–5. If both amplifications are considered (Figs 3 and 4), double amplifications may occur and, in this case, the two curves need to be evaluated by the 5-min threshold method. In particular, heterozygous samples are expected to give double amplifications with $\Delta t_{50} < 5$ min (e.g. Figure 4, samples R3, R13 and R15 of population Tu-4), resulting in being undetermined. Instead, an amplification with one primer set only (Fig. 5) results in single amplifications, which are easier to be interpreted.

A resume of the validation procedure and the estimated performance indices is reported in Table 3. Considering both amplifications, sensitivity (the ability to detect the true positives) ranged from 67% to 93%, specificity (the ability to detect the true negatives) ranged from 75% to 87%, and precision (the ability to detect the true positives avoiding the false positives) from 55% to 100%. Instead, considering only the primer set specific for the mutation, sensitivity was 100%, specificity ranged from 65% to 73%, and precision ranged from 59% to 79%. The assay performed differently depending on the species: indices were higher for *A. retroflexus* and *A. hybridus* with respect to *A. tuberculatus*. Specifically, precision appeared quite lower for *A. tuberculatus* compared to the other two species, meaning that that some plants that do not carry the mutation (and are therefore ALS-susceptible) are incorrectly counted as resistant. For *A. tuberculatus*, this index increased when only the primer set specific for the mutation was considered, whereas, for the other two species, it decreased. Instead, both sensitivity and specificity increased for all species when considering only the primer set specific for the mutation.

Resistance prediction under simulated field conditions

Thirty plants of population Hy-5 were tested for the presence of the 574-Leu mutation with the LAMP assay performed on crude genomic DNA extraction. All but one of the 30 plants had a clear positive amplification with the mutant-specific LAMP mix and most samples reached the maximum amplification between 12 and 20 min (Fig. 6), similar to that obtained during the setup and validation. Apparently, the rapid DNA extraction carried the commercial kit did not significantly affect the LAMP amplification efficiency. Only one plant (T23) resulted in a late amplification. Even when considering this plant as susceptible or not determined, all the other plants resulted in carrying the 574-Leu mutation (at least in heterozygous status) and thus a survival rate to ALS herbicides of near to 100% was expected. Indeed, the *in vivo* bioassay carried out on this population confirmed the estimation (the survival rate to both the field doses of thifensulfuron-methyl and imazamox was 100%.

DISCUSSION

The tested populations of *A. retroflexus*, *A. hybridus* and *A. tuberculatus* were shown to be cross-resistant to thifensulfuron-methyl and imazamox, confirming that these *Amaranthus* species are evolving resistance to ALS inhibitors in Italian soybean fields. These weed species were previously sometimes found at the same time in the same fields and the resistance mechanism was shown to be prevalently target site resistance as a result of a Trp-574-Leu mutation,[4] known to be dominant.[24] Therefore, it is useful to develop an accurate molecular assay that could be able to detect the mutation-endowing resistance to ALS inhibitors in these three weed species. In the present study, we developed a LAMP assay to descriminate the 574-Leu mutated plants (resistant plants) from the 574-Trp plants (susceptible plants). The CAPS assay revealed that all the ALS-resistant plants used in the setup of the LAMP assay were homozygous resistant (i.e. plants had two mutated alleles 574-Leu). The development of the LAMP assay was challanging and required the individiduation of the best set of primers and amplification parameters to achieve the

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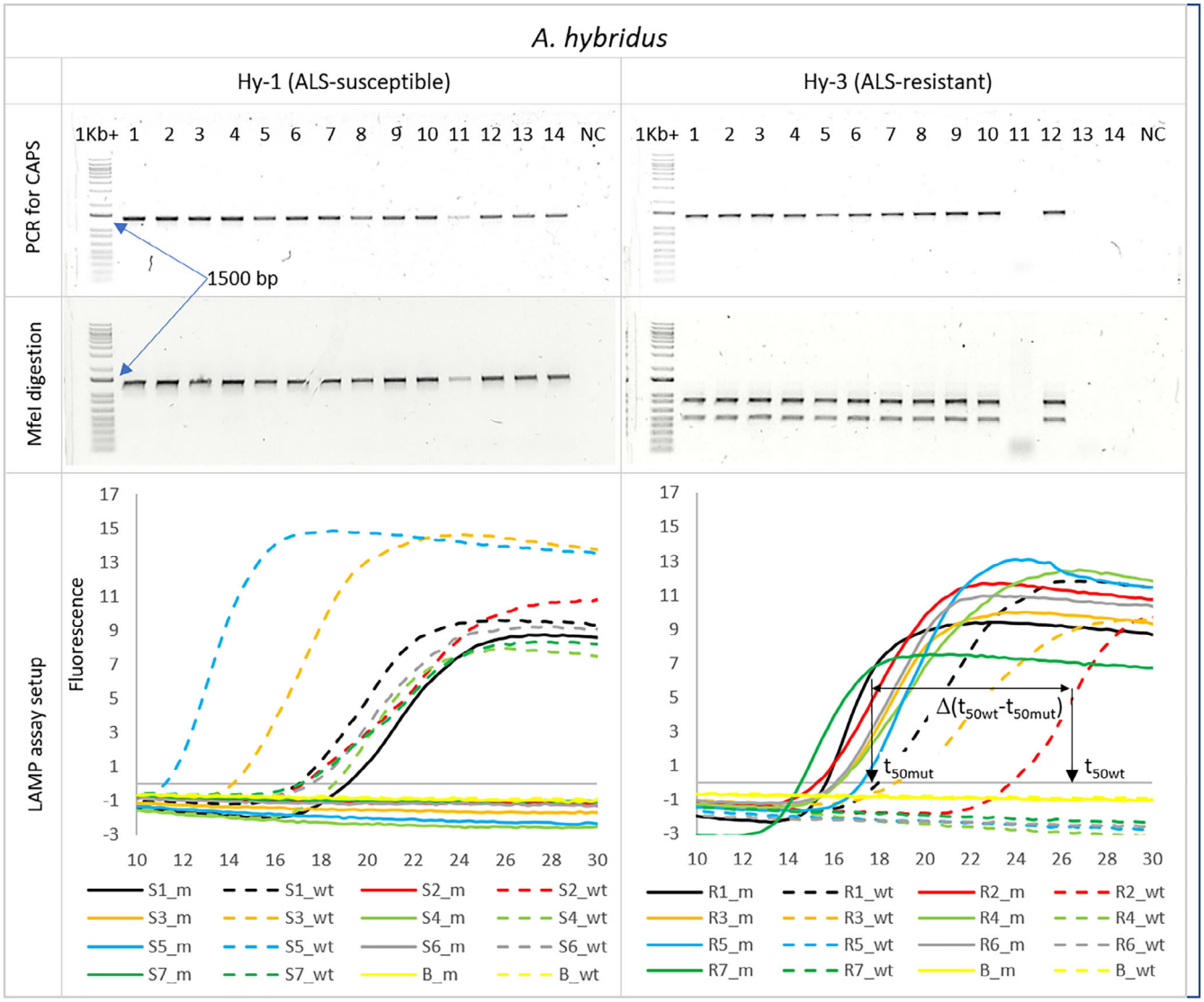

Figure 2. Example of the cleaved amplified polymorphic sequences (CAPS) (agarose gel pictures on the top) and the loop-mediated isothermal amplification (LAMP) (line graphs on the bottom) assays carried out in the setup step (*Amaranthus hybridus*). The gel pictures show the effect of the *Mfe*I enzyme digestion on PCR amplicons obtained from 14 samples of an ALS-susceptible (Hy-1, top-left) and an ALS-resistant (Hy-3, top-right) population. Line graphs report the amplification LAMP curves obtained by seven of those samples. Samples were numbered 1 to 7, S or R, depending on the resistance status of the population; no-template (water only) samples (blanks, B) are indicated in pale yellow. Each one was amplified with LAMP mixes specific for the 574-Leu mutant allele (S/R_m samples, continuous lines) and specific for the 574-Trp WT allele (S/R_wt samples, dashed lines). t_{50mut} is the time at which the reaction specific for the 574-Leu mutant allele reaches the 50% of the maximum fluorescence, whereas t_{50wt} refers to the reaction specific for the 574-Trp WT allele curve. The difference between them is indicated as $\Delta(t_{wt} - t_{mut})$.

goal. Both amplification approaches (a single primer set specific for the mutant allele, or two primer sets for both mutant and WT alleles) enabled a LAMP assay with a good balance between sensitivity (the ability to detect the true positives), specificity (the ability to detect the true negatives) and precision (the ability to detect the true positives avoiding the false positives). However, differences were observed in the LAMP assay, with both of the examined approaches, depending on the *Amaranthus* species. In particular, all the indices were more favorable for the two monoecious species, *A. retroflexus* and *A. hybridus*, despite the primers being designed on *A. tuberculatus ALS* sequence. This apparent contradiction might be justified by the higher genetic variability of a *A. tuberculatus*, which is a dioecious obligate outcrosser. The variability of the *ALS* gene has been reported to be the reason why the LAMP assay did not work in the grass weed *Bechmannia syzygachne*. This is also attributed to the fact that, being an allotetraploid organism, it has multiple copies of the *ALS* gene and this makes the design of specific primers more difficult.[16]

When only the primer set specific for the mutation was considered, the assay conducted for *A. tuberculatus* gained much in terms of sensitivity and precision, meaning that false positives (plants that do not carry the mutation but are incorrectly counted as resistant) are better discriminated from true positives. Between the two approaches, the one based on single amplification with the mutation-specific primer set is considered as the most suitable for the field application of the LAMP assay because it allows a doubling of the number of samples that can be processed per each LAMP run. For example, with the device described in the present study, only seven samples plus a blank can be processed per each run if amplifying each sample with both the primer sets.

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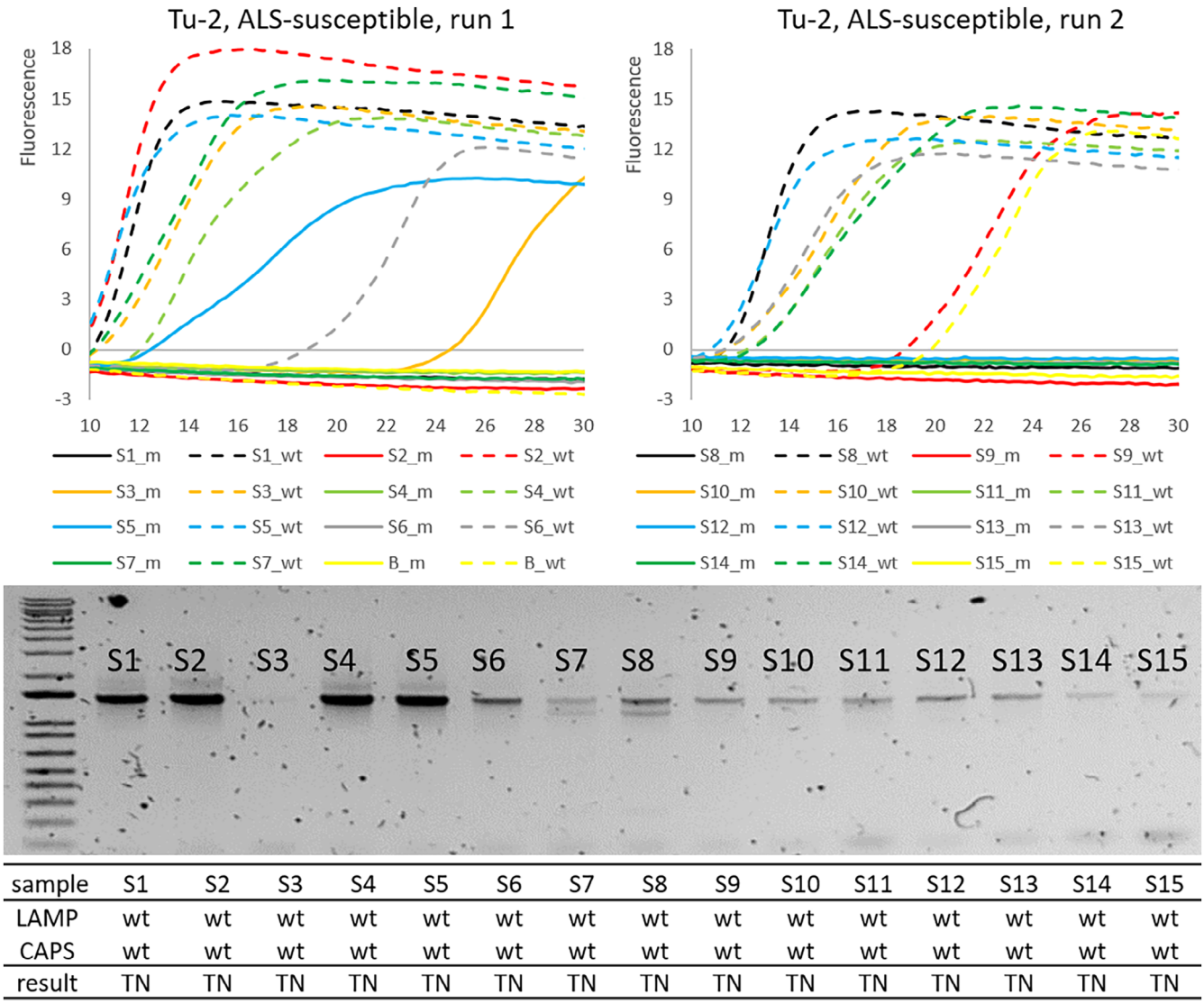

sample	S1	S2	S3	S4	S5	S6	S7	S8	S9	S10	S11	S12	S13	S14	S15
LAMP	wt	wt	wt	wt	wt	wt	wt	wt	wt	wt	wt	wt	wt	wt	wt
CAPS	wt	wt	wt	wt	wt	wt	wt	wt	wt	wt	wt	wt	wt	wt	wt
result	TN	TN	TN	TN	TN	TN	TN	TN	TN	TN	TN	TN	TN	TN	TN

Figure 3. Example of validation procedure of the loop-mediated isothermal amplification (LAMP) assay for *Amaranthus tuberculatus*. On top, line graphs report the LAMP curves obtained by 15 samples (run 1: S1→7; run 2: S8→ 15) of the susceptible population Tu-2: each sample was amplified with the mix specific for the 574-Leu mutant allele (S#_m samples, continuous lines) and that specific for the 574-Trp WT allele (S#_wt samples, dashed lines). No-template (water only) samples are indicated as 'B'. LAMP results were compared with those of the cleaved amplified polymorphic sequences (CAPS) assay (the agarose gel at the bottom) and each sample was classified as true positive (TP), true negative (TN), false positive (FP) or false negative (FN) to draw the confusion matrix. All Tu-2 samples are WT; the two bands of S7 and S8 are unspecific and not a result of the presence of the trp-574-leu allele, because the size of bands does not fit with that expected (see chapter (Detection of mutant ALS by CAPS assay) and Fig. 2).

Instead, by using only the primer set specific for the leucine allele, 15 samples plus a blank control can be processed per run. Considering that, when determining the mutated allele endowing resistance in a weed population, the sequencing on the target gene performed on 5–10 survived plants is usually considered sufficient, similarly testing 15 samples with a single LAMP run would be sufficient to obtain a robust result. Furthermore, it appears that amplification of each sample with both primer sets would generally result in higher specificity and precision but lower sensitivity, causing the underestimation of resistance. This could lead to the use of herbicides that are not effective because the resistant plants were not detected by the LAMP assay. This would potentially cause a waste of herbicides, further selection of resistance and unnecessary environmental contamination. Instead, the assay based only on the primer set specific for the mutation could overestimate the percentage of plants that are resistant to ALS. Nevertheless, a slight overestimation of resistance is not considered problematic because the consequence would be substantially limited to avoid the ALS herbicides in favor of herbicides with different modes of action. Given the immediate response of the assay, this is considered to be a very minor issue.

The described LAMP assay reveals that the amplifications performed using a crude extract of DNA instead of more purified DNA as template were similar. Therefore, combining the developed LAMP assay with this faster extraction method can speed up the protocol and facilitate on site detection of resistant plants.

Up to now, LAMP assays were successfully applied only to detect mutations endowing resistance to acetyl coenzyme A inhibitors.[16,17] To our knowledge, this is the first LAMP assay successfully applied for the detection of ALS resistance. The possibility of extending the use of this protocol to other *Amaranthus* congeneric species must be evaluated each time. Our data show

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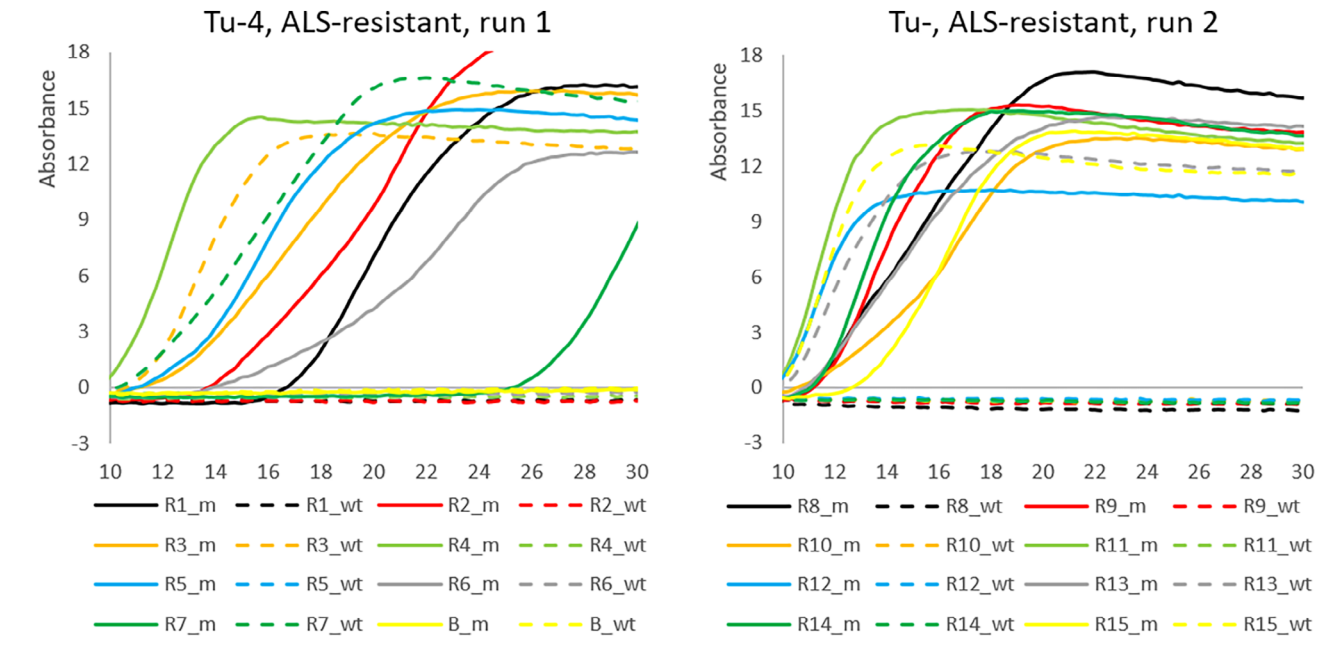

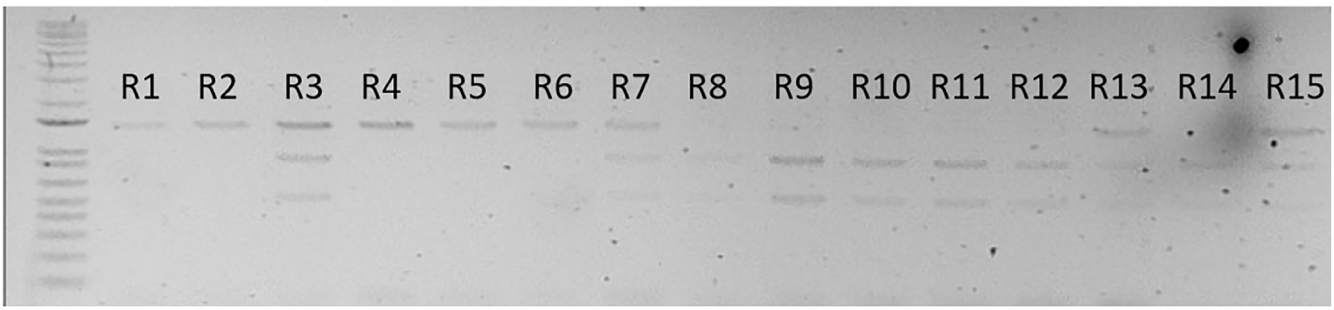

samples	R1	R2	R3	R4	R5	R6	R7	R8	R9	R10	R11	R12	R13	R14	R15
LAMP	M	M	nd	M	M	M	wt	M	M	M	M	M	nd	M	nd
CAPS	wt	wt	M	wt	wt	wt	M	M	M	M	M	M	M	M	M
result	FP	FP	nd	FP	FP	FP	FN	TP	TP	TP	TP	TP	nd	TP	nd

Figure 4. Example of validation procedure of the loop-mediated isothermal amplification (LAMP) assay for *Amaranthus tuberculatus*. On top, line graphs report the LAMP curves obtained by 15 samples (run 1: R1→7; run 2: R8→ 15) of the resistant population Tu-4: each sample was amplified with the mix specific for the 574-Leu mutant allele (R#_m samples, continuous lines) and that specific for the 574-Trp WT allele (R#_wt samples, dashed lines). No-template (water only) samples are indicated as 'B'. LAMP results were compared with those of the cleaved amplified polymorphic sequences (CAPS) assay (the agarose gel at the bottom) and each sample was classified as true positive (TP), true negative (TN), false positive (FP) or false negative (FN) to draw the confusion matrix: samples with a discrepancy between the two assays are highlighted.

that autogamous species (*A. retroflexus* and *A. hybridus*) with less intrinsic variability have a greater chance. The unsuccessful development of the LAMP assay to detect *ALS* mutations endowing resistance in *Lolium* spp. were attributed to the intrinsic genetic variability of that allogamous weed species or the target *ALS* sequence.[18,19] Nevertheless, the genetic variability is a limit also for most sequence-based detection tools.[8] Another limit of the LAMP assay is that detecting multiple mutations in a single reaction (multiplexing) is not easy because each mutation would require four primers, at least, and the risk of non-specific amplifications is high. Therefore, to detect all the possible mutations endowing ALS resistance in amaranths,[25] multiple independent LAMP reactions would be required.

Although the performance indices were estimated with well characterized populations of three amaranth species, field infestations very often consist of multiple species that are not easily distinguishable when seedlings are at the correct stage for herbicide treatment (i.e. two to four leaves stage). For this reason, if the species is not known, only the estimated average values should be considered (see the last row of Table 3). Even if it has not yet been tested, this LAMP assay might also work in other amaranth species because the primers fall in a conserved part of the gene. Despite this, because the assay is designed to specifically recognize the Trp-574-Leu allelic variants, other allelic variants cannot be detected, such as resistance that is not as a result of this point mutation (e.g. mutations in other point of the *ALS* gene).

CONCLUSIONS

The importance of accurate, very fast, in season detection of herbicide resistant populations is crucial to avoid the spread of

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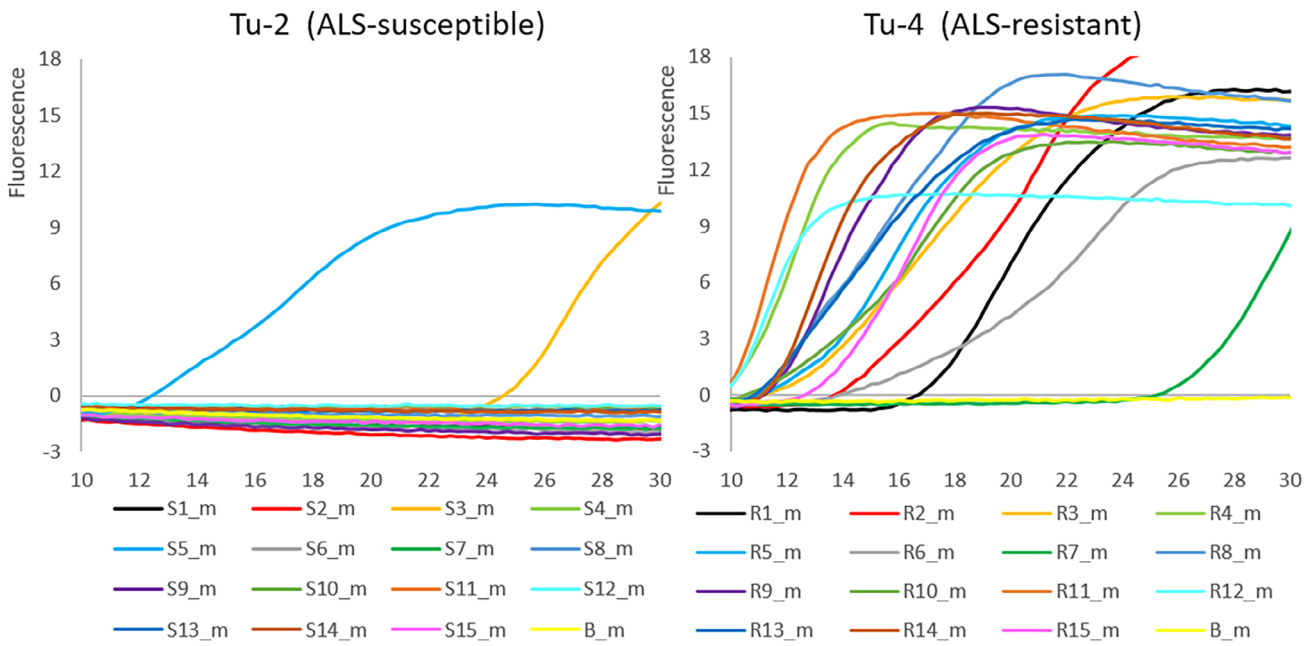

samples	S1	S2	S3	S4	S5	S6	S7	S8	S9	S10	S11	S12	S13	S14	S15	R1	R2	R3	R4	R5	R6	R7	R8	R9	R10	R11	R12	R13	R14	R15
LAMP	wt	wt	M	wt	M	wt	wt	wt	wt	wt	wt	wt	wt	wt	wt	M	M	M	M	M	M	M	M	M	M	M	M	M	M	M
CAPS	wt	wt	wt	wt	wt	wt	wt	wt	wt	wt	wt	wt	wt	wt	wt	wt	wt	M	wt	wt	wt	M	M	M	M	M	M	M	M	M
result	TN	TN	FP	TN	FP	TN	TN	TN	TN	TN	TN	TN	TN	TN	TN	FP	FP	TP	FP	FP	FP	TP	TP	TP	TP	TP	TP	TP	TP	TP

Figure 5. Validation procedure of the loop-mediated isothermal amplification (LAMP) assay of *Amaranthus tuberculatus*. Line graphs report the LAMP curves obtained by 15 samples each of population Tu-2 and Tu-4 (Tu-2: S1→15; Tu-4: R1→ 15). Each sample was analyzed considering the mix specific for the 574-leu mutant allele only. No-template (water only) samples are indicated as 'B'. LAMP results were compared with those of the cleaved amplified polymorphic sequences (CAPS) assay (Figs 3 and 4, the agarose gels at the bottom) and each sample was classified as true positive (TP), true negative (TN), false positive (FP) or false negative (FN) to draw the confusion matrix: samples with a discrepancy between the two assays are highlighted.

Table 3. Results of validation and estimation of sensitivity, specificity and precision indices for the three *Amaranthus* species and the two analyses approaches and the values averaged among species

Species	FIP primers considered for the analysis	Sensitivity (%) TP/P	Specificity (%) TN/N	Precision (%) TP/(TP + FP)
Amaranthus tuberculatus	M + WT	60	75	55
	M	100	65	59
Amaranthus retroflexus	M + WT	67	87	100
	M	100	73	79
Amaranthus hybridus	M + WT	93	87	100
	M	100	67	75
Average (if species is unknown)	M + WT	73	83	85
	M	100	68	71

Abbreviations: TP, true positive; FP, false positive; TN, true negative; FN, false negative.

resistance traits. As recently reviewed, a number of methods have been developed to detect herbicide resistance, althoug a few can be applied directly in the field and can give robust response in less than an hour.[26] In this present study, a very fast assay to detect the point mutation Trp-574-Leu of acetolactate synthase (*ALS*) in three major *Amaranthus* weed species was developed and validated. The assay worked on both DNA extracted under laboratory conditions and on DNA extracted with a commercial rapid protocol suitable for field applications. Despite still not being perfect, it is the first example of LAMP assay working on multiple weed species. Further development of this technique, or others allowing in-field analysis (e.g. Cas12a-based detection),[27] will help decision makers to promptly manage cases of resistance, aiming to better protect both the crops and the environment.

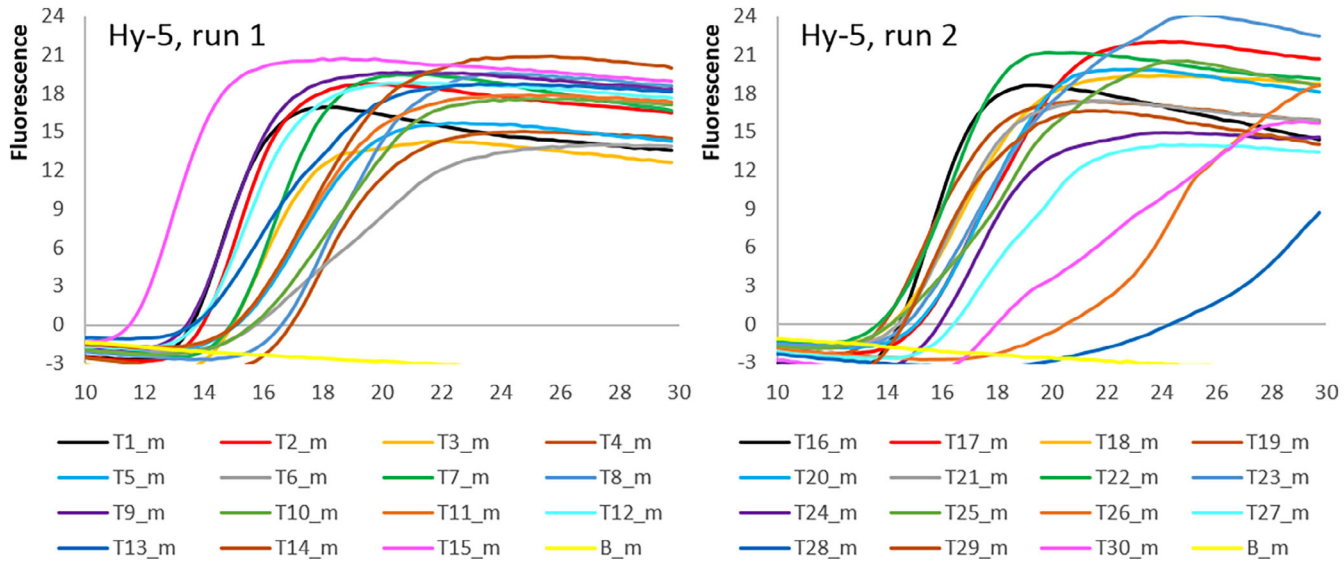

Figure 6. Results of the resistance prediction of an untested population (Hy-5) under simulated field conditions. Samples were amplified only with primers specific for the 574-Leu mutant allele (T#_m samples). No-template samples (blanks, B) are indicated in yellow.

AUTHOR CONTRIBUTIONS

AM was responsible for investigations and data curation. AM, TMG and LS were responsible for writing the original draft. SP was responsible for methodology and reviewing and editing. TMG and LS were responsible for funding acquisition. LS was responsible for conceptualization.

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CONFLICTS OF INTEREST

The authors declare that they have no conflicts of interest.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

REFERENCES

1 Heap I, The International Survey of Herbicide Resistant Weeds, 2023. http://www.weedscience.org/ [7 June 2023].
2 Singh S, Singh V, Salas-Perez RA, Bagavathiannan MV, Lawton-Rauh A and Roma-Burgos N, Target-site mutation accumulation among ALS inhibitor-resistant palmer amaranth. *Pest Manag Sci* **75**:1131–1139 (2019).
3 Iamonico D, Taxonomic revision of the genus *Amaranthus* (Amaranthaceae) in Italy. *Phytotaxa* **199**:1–84 (2015).
4 Milani A, Scarabel L and Sattin M, A family affair: resistance mechanism and alternative control of three *Amaranthus* species resistant to acetolactate synthase inhibitors in Italy. *Pest Manag Sci* **76**:1205–1213 (2020).
5 Milani A, Panozzo S, Farinati S, Iamonico D, Sattin M, Loddo D *et al.*, Recent discovery of *Amaranthus palmeri* S. Watson in Italy: Characterization of ALS-Resistant Populations and Sensitivity to Alternative Herbicides. *Sustainability* **13**:7003 (2021).
6 Travlos I, de Prado R, Chachalis D and Bilalis DJ, Editorial: herbicide resistance in weeds: early detection, Mechanisms, Dispersal, New Insights and Management Issues. *Front Ecol Evol* **8**:1–5 (2020).
7 Burgos NR, Tranel PJ, Streibig JC, Davis VM, Shaner D, Norsworthy JK *et al.*, Review: confirmation of resistance to herbicides and evaluation of resistance levels. *Weed Sci* **61**:4–20 (2013).
8 Corbett C-AL and Tardif FJ, Detection of resistance to acetolactate synthase inhibitors in weeds with emphasis on DNA-based techniques: a review. *Pest Manag Sci* **62**:584–597 (2006).
9 Délye C, Causse R, Gautier V, Poncet C and Michel S, Using next-generation sequencing to detect mutations endowing resistance to pesticides: application to acetolactate-synthase (ALS)-based resistance in barnyard grass, a polyploid grass weed. *Pest Manag Sci* **71**:675–685 (2015).
10 Cao J, Wu Q, Wan F, Guo J and Wang R, Reliable and rapid identification of glyphosate-resistance in the invasive weed *Amaranthus palmeri* in China. *Pest Manag Sci* **78**:2173–2182 (2022).
11 Kersten S, Rabanal FA, Herrmann J, Hess M, Kronenberg ZN, Schmid K *et al.*, Deep haplotype analyses of target-site resistance locus ACCase in blackgrass enabled by pool-based amplicon sequencing. *Plant Biotechnol J* **21**:1240–1253 (2023).
12 Badolo A, Okado K, Guelbeogo WM, Aonuma H, Bando H, Fukumoto S *et al.*, Development of an allele-specific, loop-mediated, isothermal amplification method (AS-LAMP) to detect the L1014F kdr-w mutation in *Anopheles gambiae* s. l. *Malar J* **11**:1–7 (2012).
13 Shen L, Huang M, Fang A, Yang Y, Yu Y and Bi C, Loop-mediated isothermal amplification for the rapid detection of the mutation of carbendazim-resistant isolates in *Didymella bryoniae*. *Agronomy* **12**:2057 (2022).
14 Hassan MM, van Vliet AHM, Higgins O, Burke LP, Chueiri A, O'Connor L *et al.*, Rapid culture-independent loop-mediated isothermal amplification detection of antimicrobial resistance markers from environmental water samples. *Microb Biotechnol* **16**:977–989 (2023).
15 Song Z, Zhang S, Man W, Li Y, Luo G, Yu J *et al.*, Rapid detection of A282S mutation in the RDL1 gene of Rice stem borer via the mutation-specific LAMP technique. *Agronomy* **13**:1–9 (2023).
16 Pan L, Li J, Zhang WN and Dong L, Detection of the I1781L mutation in fenoxaprop-p-ethyl-resistant American sloughgrass (*Beckmannia syzigachne* Steud.), based on the loop-mediated isothermal amplification method. *Pest Manag Sci* **71**:123–130 (2015).
17 Wang M, Tang Z, Liao M, Cao H and Zhao N, Loop-mediated isothermal amplification for detecting the Ile-2041-Asn mutation in fenoxaprop-P-ethyl-resistant *Alopecurus aequalis*. *Pest Manag Sci* **79**:711–718 (2023).

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18 Panozzo S, Farinati S, Sattin M and Scarabel L, Can allele-specific loop-mediated isothermal amplification be used for rapid detection of target-site herbicide resistance in *Lolium* spp.? *Plant Methods* **19**:1–13 (2023).
19 Torra J, Montull JM, Taberner A, Onkokesung N, Boonham N and Edwards R, Target-site and non-target-site resistance mechanisms confer multiple and cross- resistance to ALS and ACCase inhibiting herbicides in *Lolium rigidum* from Spain. *Front Plant Sci* **12**:1–13 (2021).
20 Manicardi A, Scarabel L, Llenes JM, Montull JM, Osuna MD, Torra Farré J *et al*., Genetic basis and origin of resistance to acetolactate synthase inhibitors in *Amaranthus palmeri* from Spain and Italy. *Pest Manag Sci* **79**:4886–4896 (2023).
21 Hess M, Barralis G, Bleiholder H, Buhr L, Eggers T, Hack H *et al*., Use of the extended BBCH scale - general for the descriptions of the growth stages of mono- and dicotyledonous weed species. *Weed Res* **37**: 433–441 (1997).
22 Doyle JJ and Doyle JL, A rapid DNA isolation procedure for small quantities of fresh leaf tissue-world vegetable center. *Focus* **12**:39–40 (1990).
23 Milani A, Scarabel L and Sattin M, *Amaranthus tuberculatus*, *Amaranthus hybridus* and *Amaranthus retroflexus* acetolactate synthase (ALS) gene, partial cds, Mendeley Data, V1 (2019). [6 February 2023].
24 Tranel PJ and Wright TR, Resistance of weeds to ALS-inhibiting herbicides: what have we learned? *Weed Sci* **50**:700–712 (2002).
25 Tranel PJ, Wright TR and Heap IM, Mutations in Herbicide-Resistant Weeds to Inhibition of Acetolactate Synthase (2023). Available: http://weedscience.org/.
26 Tataridas A, Jabran K, Kanatas P, Oliveira RS, Freitas H and Travlos I, Early detection, herbicide resistance screening, and integrated management of invasive plant species: a review. *Pest Manag Sci* **78**:3957–3972 (2022).
27 Zhang H-X, Zhang C, Lu S, Tong X, Zhang K, Yin H *et al*., Cas12a-based one-pot SNP detection with high accuracy. *Cell Insight* **2**:100080 (2023).