Cross-resistance mechanisms to ACCase-inhibiting herbicides in short-spike canarygrass (*Phalaris brachystachys*)

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Highlights

- First case of resistance due to both target-site and enhanced metabolism in ACCase-resistant of *P. brachystachys.*
- Number of resistance mechanisms can influence the resistance level.
- The ACCase Ile-1781-Thr mutation was observed in the resistant biotypes.

Abstract

Herbicides that inhibit acetyl-coenzyme A carboxylase (ACCase) are commonly used to control weedy grasses such as short-spike canarygrass (*Phalaris brachystachys*). Two resistant biotypes of *P. brachystachys* (R1 and R2) were found in different winter wheat fields in Iran. This study was done to confirm the suspected resistance observed in the field and to elucidate the resistance mechanisms involved. The results indicated that the both resistant biotypes showed cross-resistance to diclofop-methyl (DM), pinoxaden (PN) and cycloxydim (CD) herbicides. Based on the herbicide dose that inhibited 50% of the ACCase activity (I_{50}), the ACCase activity of the resistant biotypes was less sensitive than the S biotype to DM, CD, and PN. No differences in translocation were detected between biotypes; most of the herbicide remained in the treated leaves. The ^{14}C-DM metabolites were identified using thin-layer chromatography. Pre-treatment with the cytochrome P450 inhibitor ABT inhibited ^{14}C-DM metabolism in the R1 biotype, indicating that metabolism is involved in the DM resistance in the R1 biotype. DNA sequencing studies found an Ile-1781-Thr change in both resistant biotypes, conferring cross-resistance to ACCase inhibitors. In general, in the R1 biotype which showed a higher level of resistance than that of the R2 biotype, cross-resistance was observed because of mutation and DM metabolism, while in the R2 biotype, the mutation confers resistance to ACCase-inhibiting herbicides. This is the first reported evidence of the mechanisms responsible for the resistance to ACCase herbicides in *P. brachystachys*. These results could be useful for improved management of resistant biotypes carrying similar mutations.

Keywords

Cytochrome P450; Enzyme assay; Metabolism; Mutation

1. Introduction

From the beginning of agriculture, weeds have been the most harmful biotic factor reducing crop yield and quality. Weeds decrease crop yield worldwide by approximately 34% (Oerke, 2006). Herbicide use may control on average greater than 90% of weeds. Because of its effectiveness, the chemical weed control method is rapidly expanding worldwide and has become among the most widely used weed control tools (Délye, 2013). Acetyl-coenzyme A carboxylase (ACCase) inhibitors are widely used as useful tools for killing weeds.

There are two isoforms of ACCase in plants: the plastid-localized ACCase, which is essential in the biosynthesis of primary fatty acids, and the cytosol-localized ACCase, which is involved in the biosynthesis of long-chain fatty acids (Gronwald et al., 1992; Yu et al., 2007). The ACCase-inhibitors herbicides in a plastid isoform are effective in most grass weeds but have limited or no activity in a cytoplasmic isoform (Yu et al., 2007). Nearly all monocotyledonous and dicotyledonous plants have these two ACCase isoforms. The Poaceae family is an exception as similar homomeric forms found in both plastids and cytosols (Sasaki and Nagano, 2004). The homomeric ACCase in the cytosol of nearly all plant species and the heteromeric ACCase in the chloroplasts of dicotyledons are insensitive to aryloxyphenoxy propionate (APP), cyclohexanedione (CHD) and phenylpyrazoline (PPZ) herbicides (Menendez and De Prado, 1996). This difference in sensitivity to ACCase has been considered to produce a family of herbicides that selectively control grass weeds (De Prado et al., 2005). The APP, CHD and PPZ herbicides (Hofer et al., 2006) inhibit ACCase action in Poaceae, thus preventing fatty acid synthesis and reducing production of phospholipids used in membranes (Délye et al., 2002). Because of their effectiveness and convenience in grass weed management, ACCase herbicides have been used annually in various crop fields worldwide, including Iran. Repeated use of ACCase-inhibitor herbicides (APPs, CHDs, and PPZs) has resulted in the evolution of resistance in 48 grass weed species worldwide (Heap, 2019). Accordingly, ACCase inhibitor resistance has become the third most frequent type of weed resistance (Heap, 2019).

Resistance to ACCase inhibitors in grass weeds is a result of two general known groups of mechanisms: resistance caused by mutation(s) in the gene encoding the herbicide target site (target-site-based resistance: TSR) (Petit et al., 2010) and resistance resulting from a reduction in the number of herbicide molecules reaching their target such as through enhanced metabolism, foliar absorption, and translocation (non-target site resistance: NTSR) (De Prado et al., 2005; Kaundun, 2014; Huan et al., 2011). However, the most important of the NTSR mechanisms involved in ACCase-inhibitor resistance is a result of the herbicide metabolism involving a cytochrome P450 monooxygenase inhibition in grass weeds. The metabolism of ACCase inhibitors has been shown in *Alopecurus myosuroides* and *Lolium rigidum* (De Prado et al., 2005; Letouzé and Gasquez, 2003; Preston et al., 1996).

Regarding target-site resistance, different amino acid substitutions at positions 1781, 1999, 2027, 2041, 2078, 2088, and 2096 have been reported in the CT domain of the ACCase gene in various species (Powles and Yu, 2010). Amino acid substitution in these seven positions produces different resistance patterns among ACCase inhibitors. During recent years, substitutions of amino acids that lead to resistance in *P. minor* have been identified, including amino acid changes at positions 1781 (Cruz-Hipolito et al., 2015), 2027 (Gherekhloo et al., 2011), 2078 (Cruz-Hipolito et al., 2015) and 2041 (Singh et al., 2014; Raghav et al., 2016).

At present, *P. brachystachys* is among the most important grass weeds in wheat and barley fields in Iran and greatly affects crop yield. To control the grass weeds in these crops, some ACCase inhibitors have been registered in Iran during the last three decades (Gherekhloo et al., 2016). The principal aims of this study were to (1) confirm resistance to ACCase inhibitor herbicides (APPs, CHDs and PPZs) in *P. brachystachys* biotypes from Iran; (2) elucidate the mechanism behind their resistance using absorption, metabolic, and ACCase enzyme activity; and (3) determine the molecular basis for resistance to ACCase-inhibitor herbicides.

2. Materials and methods

2.1. Plant material

Seeds of resistant (R1 and R2) biotypes of *P. brachystachys* to ACCase inhibitors were collected in winter wheat fields of Golestan, Iran, during 2017. The susceptible (S) biotype originated from fields that had never been treated with herbicides. Collection information regarding the position of each biotype is listed in Table 1. Seeds were collected from 50 plants that had reached physiological maturity and were subsequently dried at laboratory temperature and then stored in paper bags in a 4 °C chamber until use during experiments.

2.2. Dose-response assay

Seeds of the R1, R2, and S biotypes of *P. brachystachys* were germinated on moistened filter paper in Petri dishes. Seedlings were planted in 8 × 8 × 10 cm pots. At the 3–4 leaf stage, the R and S plants of *P. brachystachys* were sprayed using a laboratory track sprayer equipped with a Tee Jet 8002E-VS flat-fan nozzle (Devries Manufacturing, Hollandale, MN, USA) delivering a spray volume of 300 L ha^{-1} at 200 kPa. Table 2 lists the herbicides and rates used. Four weeks after herbicide application, above-ground biomass from the plants in each pot was harvested and dried at 60 °C for 48 h.

2.3. Enzyme purification and ACCase activity assay

ACCase activity assays were completed following the methodology described by Cruz-Hipolito et al. (2012). Leaves (6 g of fresh weight) of the R1, R2 and S biotypes of *P. brachystachys* were harvested from plants during the 3–4 leaf stage. Leaf tissue was ground in liquid nitrogen in a mortar and added to an extraction buffer (24 mL) (0.1 M *N*-2-hydroxyethylpiperazine-*N'*- 2-ethanesulfonic acid-KOH (pH 7.5), 0.5 M glycerol, 2 mM EDTA, and 0.32 mM PMSF). The homogenate was mixed (3 min) using a magnetic stirrer and sequentially filtered through four cheesecloth layers. The crude extract was centrifuged (24,000×*g*, 30 min, 4 °C). The supernatant was fractionated with $(NH_4)_2SO_4$ and centrifuged (12,000×*g*, 10 min, 4 °C). The precipitate, between 35% and 45% $(NH_4)_2SO_4$ saturation, was resuspended in 1 mL of S400 buffer [0.1 M tricine–KOH (pH 8.3), 0.5 M glycerol, 0.05 M KCl, 2 mM EDTA, and 0.5 mM DTT]. The clarified supernatant was applied to a desalting column previously equilibrated using S400 buffer.

ACCase enzyme was eluted from the column in 2 mL of S400 buffer. The enzymatic activity was assayed by measuring the ATP-dependent incorporation of NaH $[^{14}C]O_3$ into an acid-stable $[^{14}C]$ product. The reaction product was previously shown to be $[^{14}C]$ malonyl-CoA. Assays were conducted in (7 mL) scintillation vials containing 0.1 M tricine-KOH (pH 8.3), 0.5 M glycerol, 0.05 M KCl, 2 mM EDTA, 0.5 mM DTT, 1.5 mM ATP, 5 mM $MgCl_2$, 15 mM NaH $[^{14}C]O_3$ (1.22 MBq μmol^{-1}), 50-µL enzyme fractions, different herbicide concentrations, and 5 mM acetyl-CoA in a final volume of 0.2 mL. Activity was assayed for 5 min at 34 °C and the reaction was stopped by adding HCl (30 µL of 4 N). A piece of filter paper was added to the reaction vial and the sample was dried (40 °C) under a stream of air. After the sample was dried, an ethanol-water mixture (1:1, *v*/*v*, 0.5 mL) was added to the vial. This process was followed by the addition of a scintillation cocktail (5 mL). Radioactivity was determined by liquid scintillation spectrometry (LSS) with a Beckman LS 6000 TA. Background radioactivity, measured as acid-stable counts (dpm) in the absence of acetyl-CoA was subtracted from each treatment. One unit of ACCase activity was defined as 1 μmol of malonyl CoA formed min^{-1}. Herbicide concentrations resulting in a 50% inhibition of enzymatic activity (I_{50} values) were estimated for each herbicide using obtained concentration response curves. The experiment was repeated twice with three replicates. Data were pooled and a non-linear regression model [Equation (1)] was fitted to the data.

2.4. ^{14}C-diclofop-methyl absorption and translocation

The R1, R2 and S biotypes of *P. brachystachys* were grown as previously described. A diclofop-methyl (DM) solution was prepared with commercial product based on the recommended dose (1 Kg a.i. ha^{-1} dissolved into 200 L ha^{-1}) and mixed with ^{14}C-diclofop-methyl (^{14}C-DM) to prepare an emulsion with a specific activity of 37.9 Bq mg^{-1} a.i. (De Prado et al., 2005; Cruz-Hipolito et al., 2012). This emulsion was applied to the surface of the second expanded leaf from each population in a 1-μL (833.33-Bq) droplet using a microapplicator (Hamilton PB 6000 dispenser, Hamilton Co., Reno, NV, USA). At 6, 12, 24, 48 and 72 h after treatment (HAT), plants were harvested in batches of three and separated into treated leaf (TL), remainder of shoots (RS), and roots (Ro). The surface of the treated leaves was washed with 1.5 mL of acetone. The washes from each batch were mixed with 2 mL of scintillation fluid (Ultima Gold ™; Perkin-Elmer, Packard Bioscience BV) and the radioactivity was determined using liquid scintillation spectrometry (LSS) (Beckman LS 6000 TA, Beckman Instruments, USA). The plant sections were individually stored in combustion cones (Combuste-Cone, Flexible: Perkin-Elmer, Packard Bioscience BV), dried at 60 °C for 48 h and combusted in a sample oxidizer (Packard 307, Packard Instruments, Meriden, CT, USA). The released $^{14}CO_2$ was trapped and counted in 18 mL of Permafluor and Carbo-Sorb E (1:1, *v/v*) (Perkin-Elmer, BV Bioscience Packard) in scintillation vials. Radioactivity was quantified using LSS and expressed as a percentage of recovered radioactivity, as in the following Equation (2):(2)% absorption =[(14C in combusted tissue)(14C in combusted tissue +14 C in leaf washes)]×100

For translocation tests, the treated (second) leaf and untreated (first and third) leaves and roots were separately harvested. The treated leaf was rinsed and measured for unabsorbed radiolabel using LSS as previously described. The treated leaf and untreated leaves and roots were oven-dried at 60 °C for 2 days, combusted in a sample oxidizer as previously described, and analyzed for radioactivity using LSS.

The translocation of ^{14}C-DM was expressed as a percent of the absorbed radioactivity. It was expressed in each part of the plant with respect to the total radioactivity present within the plant after 48 h. The experiment was repeated twice.

2.5. Phosphor imaging

A phosphor imager was used to observe ^{14}C-translocation (Cyclone, Perkin-Elmer, Packard Bioscience BV). Plants were treated with respective unlabelled and radiolabelled DM as described for the absorption and translocation assays. Whole plants were rinsed and fixed on filter paper (25 × 12.5 cm), dried at room temperature for 4 days, and then placed on a film with phosphor crystals (AGFA CURIX) for 4 h. Three plants were used for each population and the experiment was repeated twice.

2.6. ^{14}C-diclofop-methyl metabolic studies

At the three-four-leaf stage, the ^{14}C- DM metabolism was studied in the leaf tissue of two R1 and R2 and one S *P. brachystachys* biotype following the methodologies described by De Prado et al. (2005) and Cruz-Hipolito et al. (2012). Labeled herbicide was applied to the adaxial surface of the second leaf in ten droplets (0.5 µL) using a microapplicator. A total of 5000 Bq was used on each plant. Plants of the resistant and susceptible biotypes were sampled at 48 HAT. Treated leaves were washed as previously described. An aliquot of leaf wash solution was assayed for radioactivity and the remaining solution was stored (−20 °C) until analysis. The shoots from each plant were ground in liquid nitrogen using a mortar and pestle. The powder was extracted with methanol (4 mL, 80% methanol, 4 °C). The homogenate was centrifuged (20000×*g*, 20 min). The resulting pellet was washed with methanol (80%) until no further ^{14}C was extracted. The pellets were oven-dried and combusted as previously described. The supernatants were combined and evaporated to dryness (40 °C) under a stream of N_2 (10 kPa). Then, samples were redissolved in methanol (500 µL, 80%). DM and its metabolites in the supernatant were identified using thin-layer chromatography on 20 cm × 20 cm × 250 µm silica gel plates (Merck, Darmstadt, Germany; silica gel 60). A toluene/ethanol/acetic acid mixture (150/7/7; *v*/*v*/*v*) was used as the mobile phase. Radioactive zones were detected using a radio chromatogram scanner (Berthold LB 2821); their chemical nature was identified by comparing RF values to those of standards (DM, 0.70; diclofop acid, 0.44; hydroxy-diclofop, 0.34; and polar conjugates, 0.00). The experiment was performed twice with three replicates.

2.7. Metabolic study with ^{14}C-diclofop-methyl in combination with a CytP450 inhibitor

ABT is an inhibitor of CytP450 monooxygenase, the enzyme necessary for the metabolism of DM in non-toxic forms in plants (Preston et al., 1996). Individual plants of the R1, R2 and S populations of *P. brachystachys* with three leaves were collected from the pots, and the roots washed with water. Subsequently, individual plants (three plants by biotype) were placed in 50-mL containers filled with a nutrient solution containing 7.5 mg L^{-1} of ABT; a mechanism to aerate the solution was constantly used (De Prado et al., 2005). After 1 week of incubation with ABT, the second leaf of the plants was treated with ^{14}C-DM and the methodology previously described was followed to describe and quantify DM and its metabolites after 48 HAT.

2.8. DNA extraction and ACCase amplification

Total genomic DNA was extracted from fresh plants at the four-leaf stage using the Speed tools Plant DNA Extraction Kit (Biotools B&M Labs S.A., Spain) following the manufacturer'sinstructions from 100 mg of leaf material. In all cases, DNA was quantified using a NanoDrop 1000 and then immediately used for PCR or stored at −20 °C until use.

Primers were designed based on the ACCase sequence of Genbank *Phalaris minor* (accession no. AY196481) to amplify regions in the CT domain know to be involved in the sensitivity to ACCase herbicides. Two sets of primers covering all known mutation sites in region A (Ile1781 (ATA)) and region B (Trp1999 (TGG), Trp2027 (TGG), Ile2041 (ATT), Asp2078 (GAT), Cys2088 (TGT) and Gly2096 (GGC or GGA or GGT)) were designed (Table 3). Ten individual plants from each biotype were genotyped. Each DNA sample was diluted to a final concentration of 12 ng mL^{-1}. PCR was conducted in a 30 µL volume that consisted of approximately 300 ng of genomic DNA, 1.125 µL of each primer (10 pmol μL^{-1}), 3 µL of 10X Taq Green Buffer, 0.3 µL of Taq polymerase (5U μL^{-1}) (Thermo Scientific), 2.4 µL of dNTP mixture (2.5 mM), and 19.05 µL of water. The PCR was run in a Thermo cycler (T100 Thermal Cycler, BIO RAD) programmed for an initial denaturation step of 94 °C of 3 min followed by 35 cycles of 30 s at 94, 55 and 72 °C. A final extension step for 5 min at 72 °C was also included. Amplified DNA fragments were purified using the Speed tools PCR Clean-Up Kit (Biotools, B&M Labs. S.A, Spain). Two fragments of 474 and 496 bp of the CT domain of the ACCase gene corresponding to the A and B region, respectively, were sequenced by the Service of Techniques Applied to Bioscience (STAB) at the University of Extremadura.

3. Results

3.1. Dose-response assay

The calculated GR_{50} values and the resistant factor (RF) of *P. brachystachys* biotypes in response to several ACCase-inhibitor herbicides are listed in Table 4 and shown in Fig. 1. The DM resistance was confirmed in the R1 and R2 biotypes. The DM concentration that led to 50% inhibition dry weight (GR_{50}) in the S biotype was 293.87 g a.i.ha^{-1} whereas for the R1 and R2 biotypes it was 3297.68 and 2740.67 g a.i.ha^{-1}, respectively. The R1 and R2 biotypes also showed cross-resistance to cycloxydim, with GR_{50} values of 341.87 and 276.89 g a.i.ha^{-1}, respectively. The resistant biotypes also showed resistance to pinoxaden with RFs of 5.98 and 4.69 for R1 and R2, respectively. DM showed the highest RF values while those for the cycloxydim and pinoxaden herbicides were lower.

3.2. ACCase activity assay

For all herbicides, with an increase in the herbicide rate, the activity of the ACCase enzyme decreased (Fig. 2). For the R1 and R2 biotypes, the herbicide concentration necessary for 50% enzyme inhibition (I_{50}) was 7.36 and 5.89 µM for DM, 15.21 and 12.41 µM for CD, and 3.45 and 2.95 µM for PN, respectively (Table 5). Similar to the whole-plant bioassay results, the ACCase enzymatic activity on the S biotype was significantly affected at very low herbicide concentrations. The R1 and R2 biotypes of *P. brachystachys* were resistant to DM, CD and PN (Table 5). Based on these results, resistance to these herbicides in the R1 and R2 biotypes might be related to a mutation in the ACCase gene, diminishing the enzyme's sensitivity to the herbicides.

3.3. ^{14}C-diclofop-methyl foliar absorption, translocation and plant visualization

There were no significant differences in [^{14}C]-DM absorption between the resistant (R1 and R2) and S biotypes (Fig. 3). Absorption of ^{14}C-DM in the S and resistant biotypes following treatment increased. Approximately 80% of all recovered radioactivity had absorbed into the leaf tissue in the resistant (R1 and R2) and S biotypes of *P.*

brachystachys at 24 HAT. The maximum absorption was recorded at 72 HAT (Fig. 3). The resistant (R1 and R2) and S biotypes showed limited translocation of [^{14}C]-DM from treated leaves to the remainder of the plant and roots at 72 HAT. Most of the absorbed herbicide, greater than 90% (Table 6), remained intact on the leaves in all biotypes. A total of 4.97%, 6.17% and 4.73% of the herbicide translocated to the shoots in the R1, R2 and S biotypes, respectively. The [^{14}C]-DM was undetectable in roots at 72 HAT. Using phosphor imaging systems, we were able to visualize the distribution of ^{14}C-DM in the S, R1, and R2 biotypes of *P. brachystachys*. One could not detect the difference in DM translocation from the treated leaf to the root in either biotype of *P. brachystachys* from the aforementioned test, and no differences between biotypes were observed.

3.4. ^{14}C-diclofop-methyl metabolism alone and in combination with a CytP450 inhibitor

Qualitatively, the pattern of DM metabolism was similar in the resistant (R1 and R2) and susceptible (S) biotypes of *P. brachystachys.* Metabolites (DM, D-acid, and D-conjugate) were found in the R1, R2 and S biotypes (Fig. 4). The R2 and S biotypes hydrolyzed the penetrated DM to an acid form via esterase activity and remained less than 11% DM after 48 h of treatment.

The concentration of phytotoxic D-acid was 3.6 times greater in the S biotype than that in the R1 biotype at 48 HAT while there were no significant differences between the S and R2 biotypes (Fig. 4). This correlates with the kinetics of conjugate formation shown by the S and R2 biotypes, where after 48 h of DM application, the diclofop conjugate appeared at a level approximately 5.46 higher in the R1 than that in the S biotype. The D-conjugates metabolites in the R2 and S biotypes were 11.33 and 13.03, respectively. Pre-treatment with ABT solution significantly inhibited DM metabolism to polar conjugates in all biotypes assayed (Fig. 4). In the R1 biotype, the number of nontoxic conjugates formed 48 HAT decreased by greater than 80% compared to the amount observed without ABT plants. As the number of polar metabolites decreased, a significant accumulation of D-acid was observed in the R1 biotype (Fig. 4) which agreed with other experiments using CytP450 inhibitors (De Prado et al., 2005).

3.5. ACCase gene sequencing

The sequences were aligned to each other and the chloroplastic ACCase genes of other *Phalaris* populations (name accessions from GenBank). The nucleotide sequences of the A region from the resistant biotypes (R1 and R2) differed from that of the S biotype at a single nucleotide substitution (T/C), providing an amino acid substitution Ile-Thr at position 1781 (Table 7). No other mutations were found at other positions.

4. Discussion

During recent years, cross-resistance to ACCase-inhibiting herbicides in some grass weeds has become a serious threat to wheat production in Iran (Gherekhloo et al., 2016). This study examined in detail the mechanism behind the resistance of *P. brachystachys* biotypes from Iran to ACCase-inhibiting herbicides with an emphasis on absorption, translocation, metabolism, ACCase enzymatic activity, and detection of ACCase mutations endowing resistance. The *P. brachystachys* biotypes were assayed for resistance to the APP, CHD, and PPZ herbicides at the whole-plant level. Dose-response assays (Table 4) confirmed that the two biotypes were resistant to all herbicides. The resistant biotypes also showed a high resistance to DM and a low level of resistance to PN and CD (Table 4). The results herein for *P. brachystachys* agree well with those that have reported different levels of resistance and cross-resistance patterns of various grasses resistant to the three groups of ACCase-inhibiting herbicides (De Prado et al., 2005; Maneechote et al., 2005; Fernández et al., 2016; Du et al., 2016). Kuk et al. (2008) also reported the same cross-resistance pattern in Italian ryegrass (*Lolium multiflorum*). At the enzyme level, the ACCase activity of resistant biotypes was much less sensitive to DM and CD and slightly less sensitive to PN (Table 5). Clearly, the resistant biotypes showed a certain level of target site cross-resistance to ACCase-inhibiting herbicides. This result suggests that a less sensitive form of the target enzyme confers cross-resistance to ACCase-inhibiting herbicides in the studied *P. brachystachys* biotypes. Cruz-Hipolito et al. (2012) reported that ACCase-herbicide resistance in *P. paradoxa* correlates with reduced sensitivity in the target enzyme.

In the present study, differences in herbicide absorption and translocation of C^{14}-DM between the resistant (R1 and R2) biotypes and S biotype were not significant, suggesting that differential absorption and translocation do not contribute to resistance to this herbicide in these *P. brachystachys* biotypes. Previous research has demonstrated that differences in foliar uptake between resistant and susceptible biotypes have not been involved in *Poaceae* weed resistance to DM, our results agree well with those of previous studies that reported grasses show limited translocation of DM (Liebl and Worsham, 1987; De Prado et al., 2005). These data suggest that resistance is not relevant to differential DM absorption and translocation.

DM metabolism was quantitatively and qualitatively similar in the R2 and S biotypes but not in the R1 biotype. The results herein showed that an enhanced metabolism mediated by CytP450 monooxygenase plays a role in DM resistance in the R1 biotype. In agreement with other research studies that used CytP450 inhibitors (amitrole and ABT), the number of nontoxic D-conjugates formed at 48 HAT in the R1 biotype was 7 times less than the number of D-conjugate metabolites compared to that of the R1 biotype without ABT. Unlike the absorption and translocation results, the altered herbicide metabolism is a non-target site mechanism responsible for the resistance in the R1 biotype of *P. brachystachys*. There are few cases in which metabolism has been described as being the dominant mechanism of ACCase resistance. Many compounds such as ABT, amitrole, PBO, and malathion are effective in inhibiting herbicide metabolism in plants catalyzed by CytP450. Studies clearly have shown that DM is rapidly de-esterified by hydrolysis in plants and forms D-acid, which is the main active ingredient (De Prado et al., 2005; Yu et al., 2007). Then, D-acid is metabolized to sugar conjugates of hydroxy-diclofop, which is mediated by CytP450 monooxygenases; this compound is more polar than D-acid and is not phytotoxic (Kaundun, 2014).

Resistance resulting from target gene mutation has been extensively studied throughout the world (Heap, 2019). Target site resistance is primarily caused by an amino acid change in the CT domain, which affects the effective binding of the ACCase inhibitor (Powles and Yu, 2010). Here, we identified an Ile-1781-Thr substitution in the resistant biotypes (R1 and R2) from the conferred resistance to the APP and CHD herbicides as well as to pinoxaden (PPZ). Until now, the mutation at position 1781 has been identified as the most common mutant resistant ACCase isoform and biotypes with this mutation have been shown to be resistant to APP, CHD and PPZ (Yu et al., 2007; Délye et al., 2008). The crystal structures of the ACCase yeast CT domain in complex with ACCase inhibitors indicate that position 1781 is in a binding pocket that is occupied by both methyl or ethyl groups by ACCase inhibitors; as a result, the 1781 mutation can confer cross-resistance to all three herbicide classes (Yu et al., 2007). In general, three allelic variants have been identified at position 1781 to date. The Ile replacement by the three different amino acids of Leu, Val, and Thr has been reported in weeds (Murphy and Tranel, 2019). The Ile-1781-Val mutation has been reported to confer at best relatively low resistance levels to some ACCase herbicides in a *P. paradoxa* population (Collavo et al., 2011). The Ile-1781-Thr mutation has only recently been discovered in a black-grass (*Alopecurus myosuroides* Huds) population (Kaundun, 2014). Resistance to ACCase-inhibiting herbicides in *Phalaris* spp. has already been described in *P. paradoxa* (Collavo et al., 2011, Hochberg et al., 2009) and *P. minor* (Gherekhloo et al., 2011; Cruz-Hipolito et al., 2015). The relationship between the mutation position and cross-resistance pattern has been established for the R1 and R2 biotypes. Furthermore, this mutation might be responsible for cross-resistance to three ACCase family groups. A point mutation in the ACCase gene (target site) contributed to resistance in *P. brachystachys*. The addition of enhanced metabolism (non-target site) could increased the resistance level depending on the enhanced rate of diclofop-methyl metabolism in the R1 biotype compared to that of the R2 biotype. The target-site and enhanced-metabolism mechanisms have been shown in *L. rigidum*, *A. sterilis* and *C. echinatus* (Tardif and Powles, 1994; Maneechote and PrestonPowles, 1997; Han et al., 2015; Fernández et al., 2016).

In this research, the *P. brachystachys* biotypes were resistant to ACCase-inhibiting herbicides. The cross-resistance observed in the R2 biotype was a result of a mutation while the resistance in the R1 biotype was associated with an enhanced metabolism mediated by CytP450 monooxygenase and mutation at the CT domain of the ACCase gene (Ile-1781-Thr). However, compared to target-site resistance, non-target-site herbicide metabolic resistance is more economically threatening because it can confer a wide range of resistance to unique herbicides and even non-commercial herbicides. In particular, metabolic herbicide resistance is a major threat to herbicide efficacy and crop productivity and warrants considerable research and management.

5. Conclusion

In conclusion, we documented the evolved resistance to diclofop-methyl and cross-resistance to cycloxydim and pinoxaden in *P. brachystachys* biotypes from wheat fields in Iran. The reason of Accase-inhibiting herbicide resistance is its use for more than 15 years. In this study, a point mutation in the ACCase gene (target site) contributed to resistance in *P. brachystachys*. The addition of enhanced metabolism (non-target site) increased the resistance level depending on the enhanced rate of diclofop-methyl metabolism in the R1 biotype compared to that of the R2 biotype. The R2 biotype without non-target site resistance had a lower RF in the greenhouse experiments than that of the R1 biotype. Most likely, the limited grass herbicide options for wheat crops in Iran and the lack of crop rotation may have aided the selection of target site mutations in *P. brachystachys* and the consequent resistance to ACCase inhibitors. This study is the first documented case in the world in which two mechanisms (TSR and NTSR) are involved in cross-resistance to ACCase-inhibiting herbicides in *P. brachystachys*.

Contributions

Sajedeh Golmohammadzadeh, Antonia M. Rojano-Delgado, Jose G. Vazquez-Garcia, Yolanda Romano, Maria D. Osuna, Javid Gherekhloo and Rafael De Prado were responsible for design of and conducting the experiments, planning, analysis of data and preparation of the manuscript.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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TABLES AND FIGURES

Table 1. Geographical location, collection site of *P. brchystachys* biotypes.

Biotypes	**Geographical location**		**Collection site**
	Province	**Coordinate point**	
R1	Golestan	(54.44°N, 36.57°E)″	Wheat field
R2	Golestan	(54.47°N, 36.53°E)″	Wheat field
S	Golestan	(54.25°N, 36.48°E)″	Wheat field

Table 2. Herbicide treatments applied for dose–response assays.

Herbicides	**Rate (g a.i. ha^{-1})**		**Field recommended Dose (g a.i. ha^{-1})**
	Biotype S	**Biotypes R**	
Diclofop-methyl (DM)	0, 72, 144, 250, 360, 500, 720, 1000, 1440	0, 1000, 1500, 3000, 3500, 4000, 5000, 6000	900
Cycloxydim (CD)	0, 20, 40, 60, 100, 200,400	0, 100, 200, 300, 400, 600, 800, 1000	90
Pinoxaden (PN)	0, 4, 8, 16, 32, 64, 124, 250	0, 25, 50, 100, 150, 200, 400, 600, 800	150

Dry biomass data were expressed as a percentage with respect to the untreated control for each biotype. Herbicide rates that inhibit plant growth by 50% compared to that of the untreated control (GR_{50}) were determined for each biotype and the R/S ratio was computed as $GR_{50}(R)/GR_{50}(S)$ (Cruz-Hipolito et al., 2012). The data were pooled and fitted to a nonlinear, four-parameter logistic regression model with the upper asymptote fixed at 100%, as in Equation (1), using “R” with the “drc plug-in” statistical software (Ritz and Streibig, 2005).(1)$y=c+d-c1+(\exp\{b(\log(x)-\log(e))\}$

In the model, y is the fresh above-ground weight expressed as a percentage of the untreated control; c and d are the coefficients corresponding to the lower and upper asymptotes, respectively; b is the slope of the line; g is the herbicide rate at the point of inflection halfway between the upper and lower asymptotes; and x (independent variable) is the herbicide dose.

Table 3. Primers used for region A and B.

Primer	Sequence (5′-3′)	Assay
CRUSS-F	GATTGGCATAGCCGATGAAG	Region A
CRUSS-R	TGGACAACACCATTGGTAGC	Region A
AC6F	AGCTTGGAGGAATCCCTGTT	Region B
AC6R	GGGTCAAGCCTACCCATACA	Region B

Table 4. Parameters of the log-logistic equations used to calculate the herbicides rates (g ai ha^{-1}) required for 50% reduction dry weight (GR_{50}) of *P. brachystachys* biotypes.

Herbicide	**Biotype**	**d (SE)**	**b (SE)**	**P-value**	**R^2**	**GR_{50} (SE)**	**RF**
DM	R1	100.57 (1.35)	0.48	<0.0001	0.90	3297.68	11.22
	R2	100.65 (3.01)	0.76	<0.0001	0.99	2740.67	9.32
	S	101.77 (1.59)	0.32	<0.0001	0.98	293.87	–
CD	R1	101.61 (2.37)	0.53	<0.0001	0.98	341.87	5.79
	R2	101.04 (2.81)	0.56	<0.0001	0.98	276.89	4.69
	S	102.81 (3.30)	0.45	<0.0001	0.98	58.98	–
PN	R1	100.73 (2.64)	0.56	<0.0001	0.95	346.99	5.98
	R2	100.48 (2.94)	0.62	<0.0001	0.99	289.86	4.98
	S	101.6 (2.51)	0.65	<0.0001	0.96	57.98	–

Table 5. Parameters of the log-logistic equations used to calculate herbicide concentration required to reduce 50% of the ACCase activity (I_{50}) of *P. brachystachys* biotypes and the resistance factor (RF) of resistant biotypes.

Herbicide	**Biotype**	**d (SE)**	**b (SE)**	**P-value**	**R^2**	**I_{50} (SE)**	**RF**
DM	R1	100.77 (1.79)	0.52 (0.05)	<0.0001	0.98	7.36 (0.09)	27.9
	R2	101.50 (1.85)	0.48 (0.04)	<0.0001	0.98	5.89 (0.11)	20.7
	S	101.65 (2.01)	0.64 (0.04)	<0.0001	0.97	0.28 (0.005)	–
CD	R1	103.41 (3.30)	0.51 (0.07)	<0.0001	0.98	15.21 (0.92)	21.4
	R2	103.11 (93.37)	0.52 (0.07)	<0.0001	0.97	12.43 (0.89)	17.5
	S	100.04 (3.81)	0.60 (0.08)	<0.0001	0.96	0.71 (0.03)	–
PN	R1	100.96 (2.51)	0.53 (0.08)	<0.0001	0.97	3.45 (0.3)	9.8
	R2	101.43 (2.64)	0.49 (0.07)	<0.0001	0.98	2.95 (0.76)	8.5
	S	101.48 (2.94)	0.54 (0.05)	<0.0001	0.97	0.32 (0.009)	–

Table 6. Radiolabel translocation from the treated leaf in resistant and susceptible biotypes of *P. brachystachys* 72 HAT with [^{14}C]-DM.

Biotype	Translocation (% of absorbed)		
	Treated leaf	Shoots	Roots
R1	95.03 (1.31) a	4.97 (0.23) a	nd
R2	93.83 (1.59) a	6.17 (0.40) a	nd
S	95.27 (1.45) a	4.73 (0.52) a	nd

Table 7. Partial nucleotide and deduced amino acid sequence alignment of ACCase gen from S, R1, R2 *P. brachystachys* biotypes. Shaded amino acids indicate Ile (ata) to Thr (aca) at position 1781.

Amino acid location	1779	1780	1781	1782	1783
Consensus sequence	Glu	Asn	Ile	His	Gly
	gag	aac	ata	cat	gga
***Phalaris minor* (AY196481)**	Glu	Asn	Ile	His	Gly
	gag	aac	ata	cat	gga
***Phalaris brachystachys* (S)**	Glu	Asn	Ile	His	Gly
	gag	aac	ata	cat	gga
Phalaris brachystachys (R1)	Glu	Asn	**Thr**	His	Gly
	gag	aac	**aca**	cat	gga
Phalaris brachystachys (R2)	Glu	Asn	**Thr**	His	Gly
	gag	aac	**aca**	cat	gga

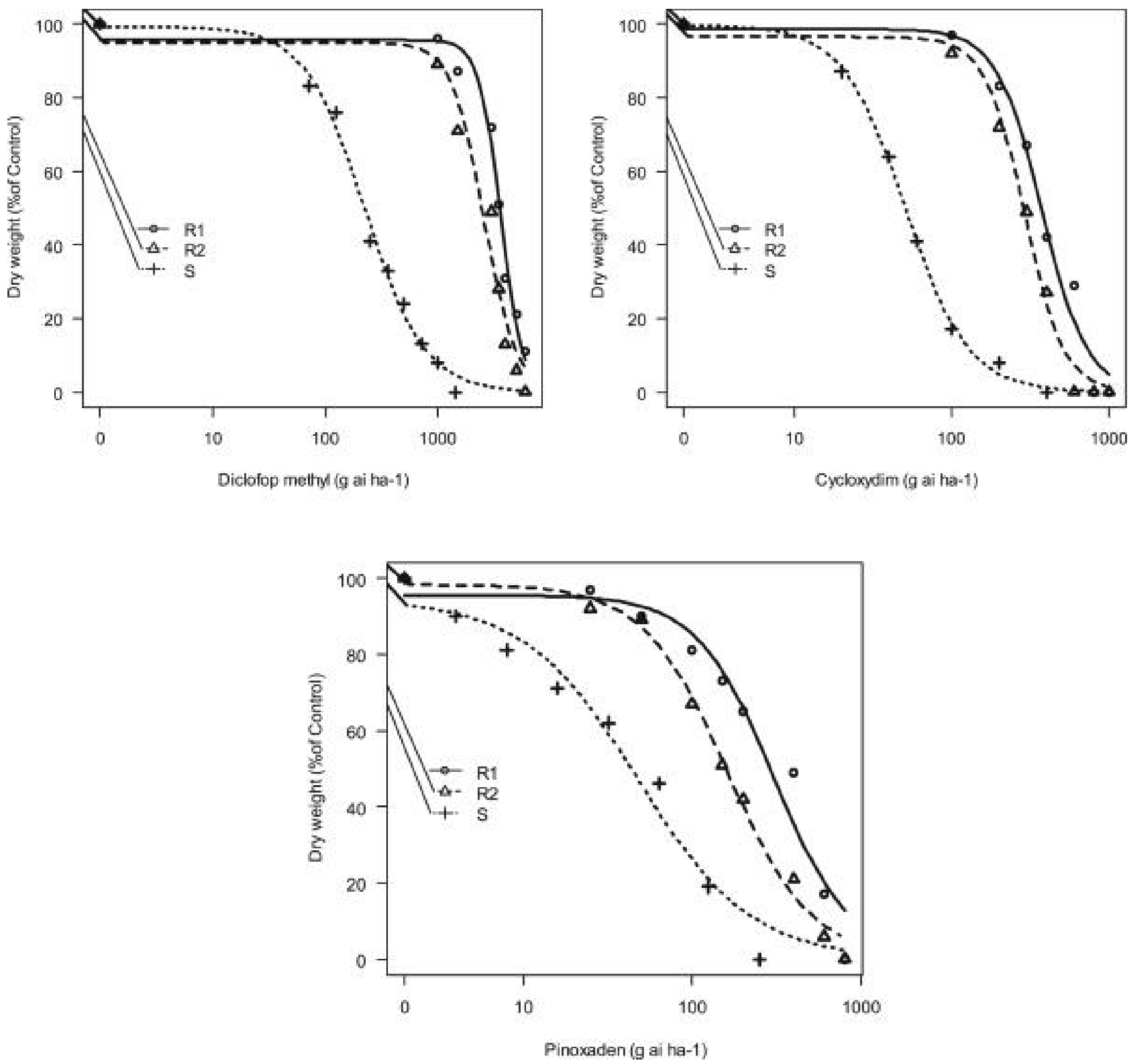


Fig. 1. Response of dry weight of susceptible (S) and resistant (R1 and R2) biotypes of *P. brachystachys* to different doses of the diclofop-methyl, cycloxydim and pinoxaden.

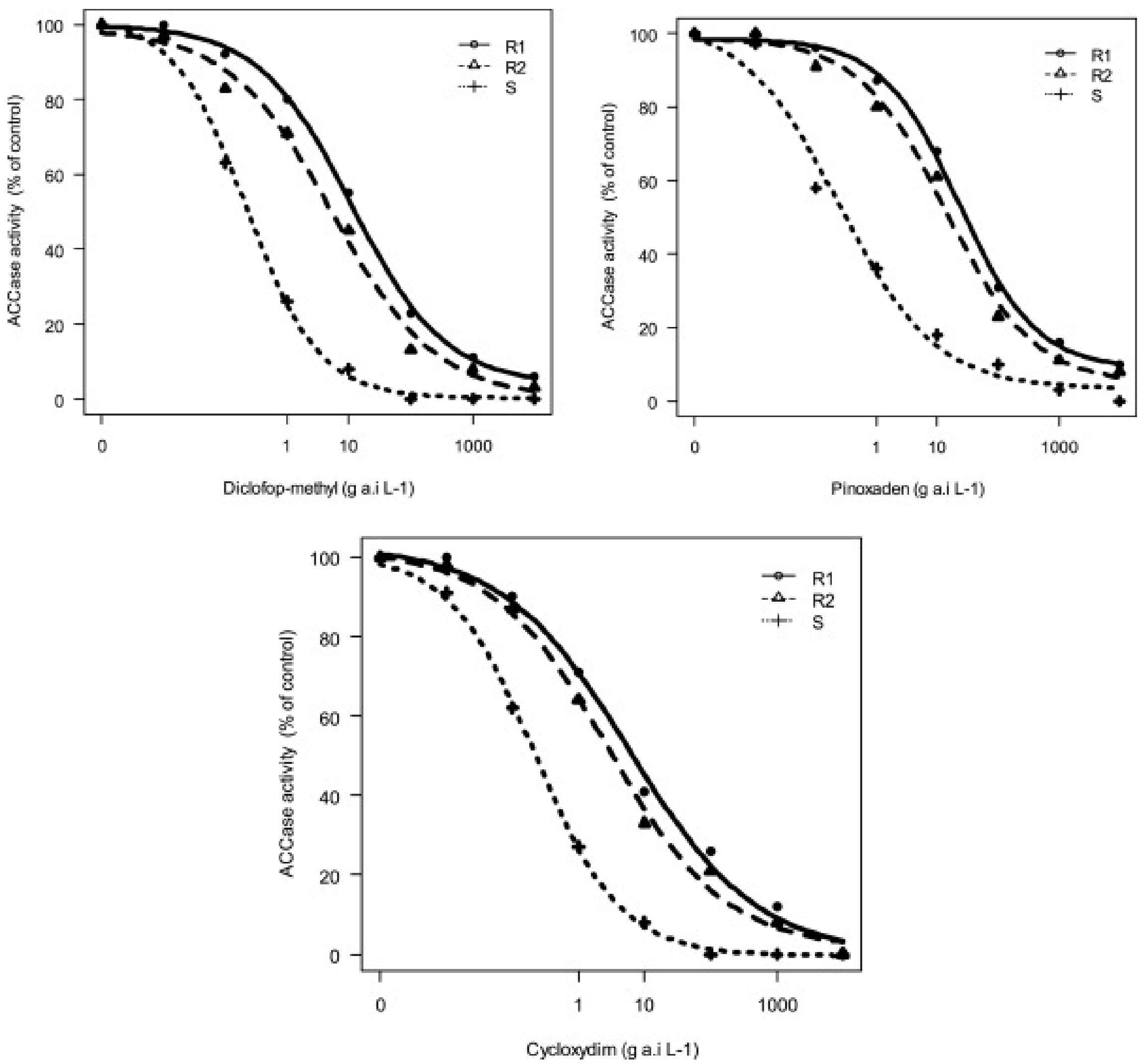


Fig. 2. Effects of ACCase inhibitor herbicides (diclofop-methyl, cycloxydim and pinoxaden) on ACCase activity of resistant (R1 and R2) and susceptible (S) *P. brachystachys* biotypes expressed as a percentage of the untreated control.

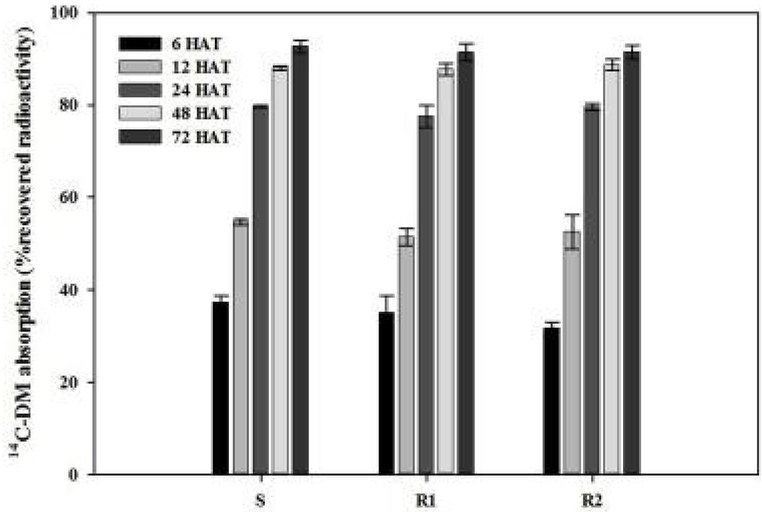


Fig. 3. Absorption of 14C-DM in *P. brachystachys* biotypes:susceptible (S) and resistant (R1,R2).

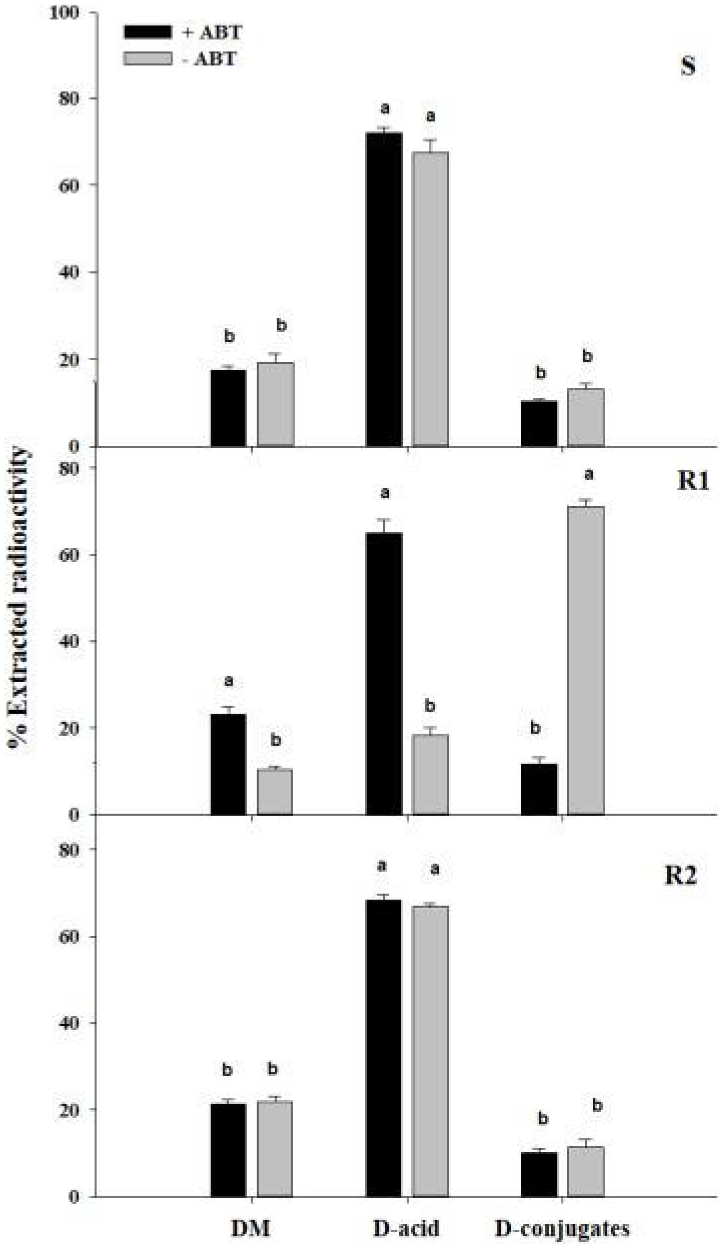
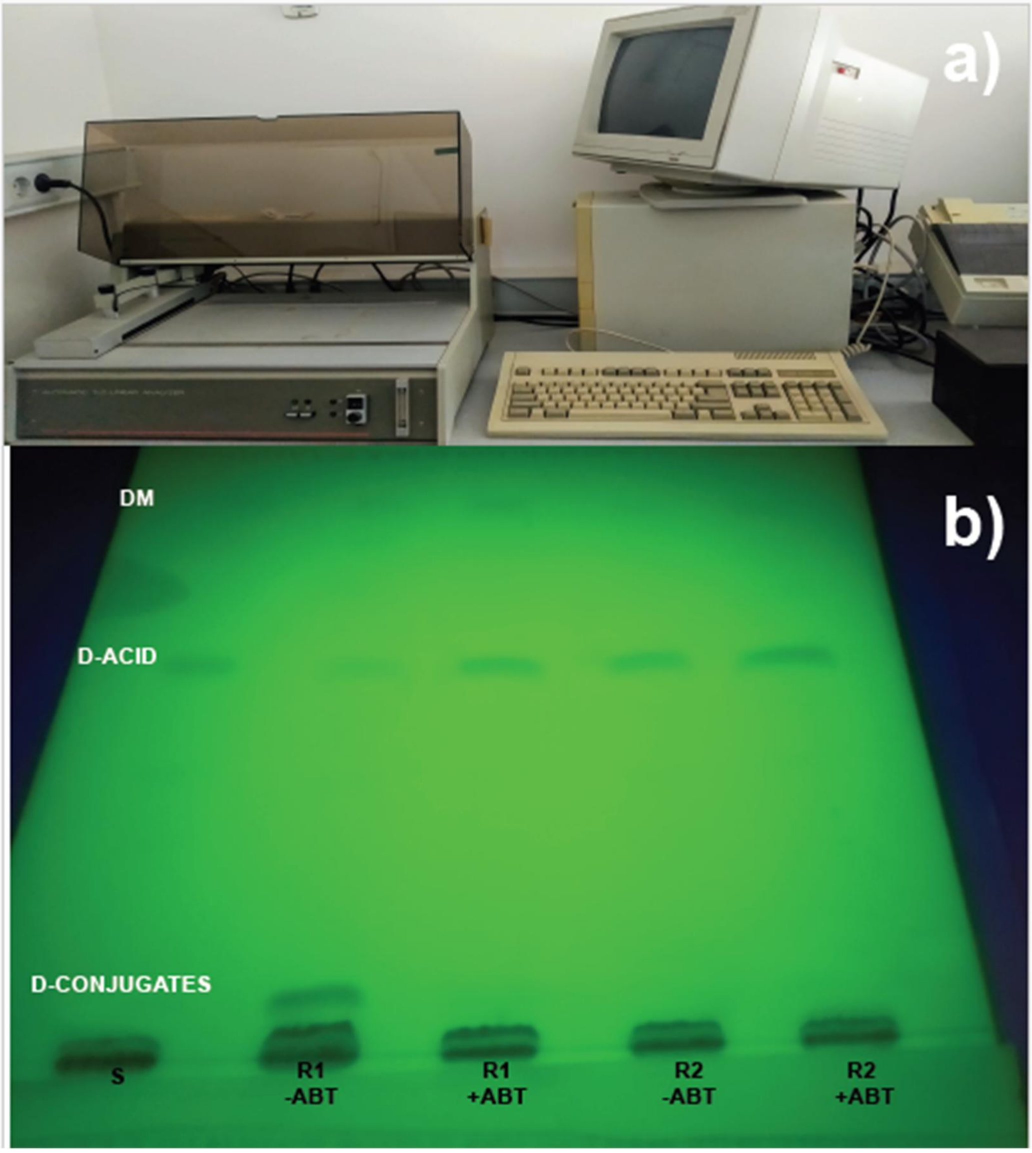
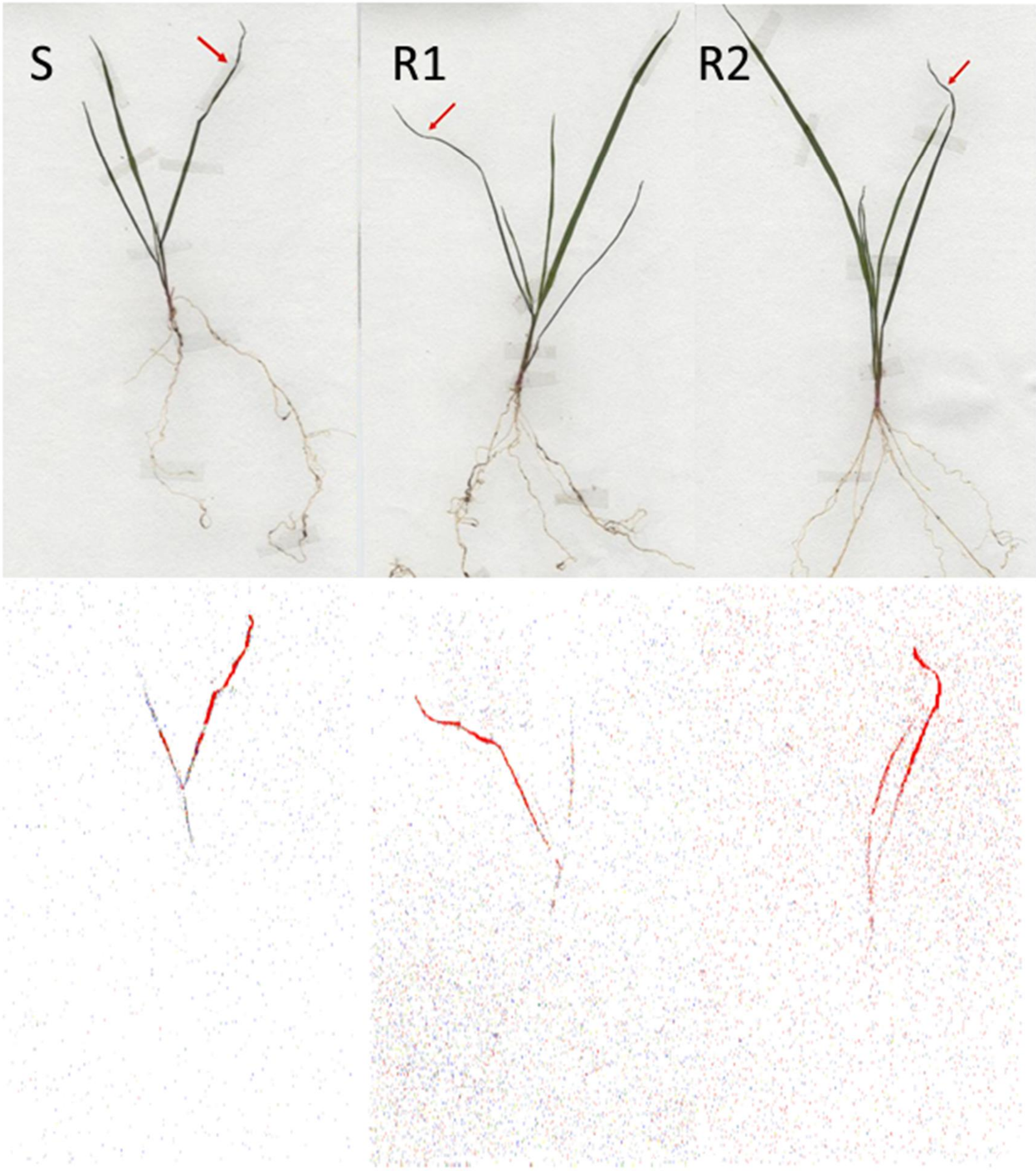


Fig. 4. Relative percentage of ^{14}C-DM and its metabolites of different resistant (R) and susceptible (S) *P. brachystachys* biotypes 48 HAT.

SUPPLEMENTARY DATAS



Suppl Figure 1. a) Used TLC equipment; b) Samples on the plate under UV light showing the different metabolites.



Supplementary Figure 2. Phosphor images showing movement of 14C-DM in S, R1 and R2 P. brachystachys biotypes. Images were recorded 48 HAT; the intensity of red coloration indicates higher concentrations of 14C.