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QUICK BIOASSAY TEST FROM TILLERS FOR DETECTING ALS HERBICIDE RESISTANCE OF WEEDY RICE AND BARNYARDGRASS

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Abstract

Resistance to acetolactate synthase (ALS) inhibitors have increased recently in South Brazil where the major weeds of flooded rice (barnyardgrass and weedy rice) have evolved resistance to imazapyr+imazapic. The aim of this research was to evaluate a growth medium for tissue regeneration of tillers in barnyardgrass, as well as an agar-based bioassays test (also from tillers) to detect susceptible and resistant biotypes of weedy rice and barnyardgrass to imazapyr+imazapic in vitro. Greenhouse experiments were conducted to detect ALS-resistant (R) and susceptible (S) weedy rice and barnyardgrass biotypes, and bioassays were carried out to evaluate an adequate growth medium for barnyardgrass tiller regeneration and determine the concentration of herbicide to distinguish R and S plants. The culture medium that provided a suitable barnyardgrass growth was MS 50% with the addition of benzylamino-purine. The tissue regeneration in vitro with the growth medium containing imazapyr+imazapic allowed to discriminate between R and S barnyardgrass and weedy rice plants. The concentration required for satisfactory control of susceptible barnyardgrass and weedy rice explants grown in vitro was 0.9 µM and 1.3 µM of imazapyr+imazapic herbicide, respectively. The bioassay in vitro using tiller regeneration provides an opportunity to predict effectively imazapyr+imazapic resistance in barnyardgrass and weedy rice.

Keywords: BAP. *Echinochloa* spp.. Growth medium. *Oryza sativa*. Tiller regeneration.

1. Introduction

Rice (*Oryza sativa* L.) is one of the most widely grown cereal crops around the world. In Brazil, the Rio Grande do Sul State represents 70% of the national production (CONAB 2020). In this region rice is grown in paddy fields with average yield of 8.3 kg ha^{-1}, while the world average yield is 4.6 kg ha^{-1} (CONAB 2020; FAO 2020). In most areas, rice is grown almost exclusively, as a reason of soil limitations based on the physical characteristics that trigger water deficit very likely, either by flooding or drought (Bueno et al. 2020). Weed infestation is among the major constraints for sustainable rice production in the state, once weeds compete for the availability of resources, as well as acting as host for insect pests and diseases (Avila et al. 2021).

The most important weeds in Rio Grande do Sul rice production are weedy rice (*Oryza sativa*) and barnyardgrass (*Echinochloa* spp.) (Fruet et al. 2020). Weedy rice belongs to the same species as cultivated rice and have similar anatomical and physiological traits (Piveta et al. 2021), which can result in greater losses due to having similar requirements for survival (Ziska et al. 2015). Barnyardgrass and weedy rice are the most important weeds in paddy rice worldwide, since they are well adapted to flooded environments, with yield loss potential up to 99% when the crop was maintained in the competition until harvest (Nadir et al. 2017; Agostinetto et al. 2021). If weeds in rice cannot be controlled, they could adversely affect food security as well as the global economy (Nadir et al. 2017).

Herbicides are commonly used for controlling weeds in crop production systems. In 2003, imidazolinone-tolerant rice varieties were introduced, providing a valuable tool for weedy rice control, changing the production system. These herbicides have a broad weed control spectrum, made possible to cultivate rice in areas where weedy rice was present in extremely high infestations (Merotto Jr. et al. 2016). However, repeated and extensive use of the same herbicide or different herbicides with the same site of action over time has increased the number of herbicide resistance unique cases worldwide (Moss et al. 2019). In Brazil, barnyardgrass has evolved resistance to cellulose, ALS, ACCase and EPSPs inhibitors (Heap 2021), as well as weedy rice to ALS inhibitors (Ulguim et al. 2021). Resistance to ALS inhibitors can be due to target site mutation and/or detoxification via enhanced metabolism. Metabolism is more complex involving multiple CYP450 and glutathione S-transferase (GST) enzymes in herbicide detoxification (Gaines et al. 2020).

The diagnosis of resistance of uncontrolled weeds or its confirmation prior to herbicide application are information required for a decision-based management that can be used on the current or in the next crop season. Early confirmation of herbicide resistance may reduce costs by reducing the number of herbicide applications, especially when effective herbicides are available (Kaundun et al. 2011). Several methods have been developed for confirming herbicide resistance. The most commonly used method (conventional method) is based on the evaluation of plant response to increasing herbicide rates sprayed on plants grown in greenhouse or in the field (Burke et al. 2006; Kaundun et al. 2011). Alternatively, resistance can also be identified by enzyme assay or molecular markers (Roso et al. 2010). The resistance confirmation by conventional, molecular or biochemical methods is generally labor-consuming and expensive (Roso et al. 2010). These limitations could delay the identification of resistance, or even difficult to process a large number of samples, especially when the results are required in a short period of time.

Quick tests for detection of weed resistance have been developed in Petri dishes, germination in paper towel, growth medium containing agar, hydroponic culture, using seeds, seedling, pollen or parts of a plant (Burke et al. 2006; Kaundun et al. 2011). The resistance of *Lolium* spp. to ALS and ACCase inhibitors herbicides were evaluated by agar bioassay in seedling growth, and 10 days after the herbicide treatment this test made possible the identification of resistance (Kaundun et al. 2011). For a quick detection of herbicide resistance at the same growing season in order to choose the fastest decision of control management, the use of tissue culture by the evaluation of tillers may be a viable alternative, once that few survivor plants available in the field may be checked in short time to confirm herbicide resistance.

Plant tissue culture involves the growth of plant cells and tissues in vitro in a microbe-free environment (Jelenska et al. 2000). Differentiated organs such as roots and shoots can be propagated in vitro. Because they grow relatively quickly, a fast response to xenobiotics such as herbicides, can be reached. However, the medium type and concentration, as well as, the use of phytohormones may affect the explant growth. In this work we detected ALS resistant and susceptible biotypes of weedy rice and barnyardgrass from rice fields in the southern Brazil using the conventional method to confirm herbicide resistance. The aim of this research was to evaluate an adequate growth medium for tissue regeneration from tillers, as well as, discriminating rates of imazapyr + imazapic included in growth medium to detect between susceptible and resistant biotypes of weedy rice and barnyardgrass at agar-based bioassays as a quick test.

2. Material and Methods

Barnyardgrass and weedy rice screening to confirm ALS resistance

Samples of barnyardgrass and weedy rice seeds were collected from rice fields of Rio Grande do Sul State in which plants had survived to application of ALS-inhibiting herbicides. Each collection point was identified by the Global Positioning System (GPS) (Table 1), and seeds were collected from a single plant. A total of 17 seeds samples of barnyardgrass were collected in the counties of Arroio Grande, Cachoeirinha and Santa Vitória do Palmar and seven samples of weedy rice in Arroio Grande, Cachoeirinha e Cachoeira do Sul were collected. The seeds then were cleaned, identified with their global positioning and stored in a cold chamber (15 °C).

Table 1. Geographic location and response at 28 days after treatment of barnyardgrass and weedy rice biotypes to the spray of imazapyr+imazapic.

Biotype	Municipalities	Geographic coordinates		imazapyr+imazapic response
		Latitude	Longitude	
Barnyardgrass				
CSA	Cachoeirinha	29° 57’ 05’’	51° 06’ 50’’	0[1]
CSB	Cachoeirinha	29° 57’ 03’’	51° 07’ 01’’	0
CSC	Cachoeirinha	29° 57’ 05’’	51° 06’ 50’’	0
CSC5	Cachoeirinha	29° 57’ 05’’	51° 06’ 50’’	0
CS2	Arroio Grande	32° 19’ 23’’	52° 57’ 34’’	0
CS7	Arroio Grande	32° 19’ 20’’	52° 57’ 22’’	0
CS8	Arroio Grande	32° 19’ 15’’	52° 57’ 04’’	0
CR1	Arroio Grande	32° 19’ 05’’	52° 57’ 44’’	0
CR2	Arroio Grande	32° 19’ 53’’	52° 57’ 14’’	0
CR3	Arroio Grande	32° 19’ 55’’	52° 57’ 54’’	0
CR4	Arroio Grande	32° 19’ 03’’	52° 57’ 10’’	1
CR6	Arroio Grande	32° 19’ 12’’	52° 57’ 09’’	1
CR8	Arroio Grande	32° 19’ 11’’	52° 57’ 17’’	1
CR9	Arroio Grande	32° 19’ 29’’	52° 57’ 47’’	0
CR10	Arroio Grande	32° 19’ 09’’	52° 57’ 16’’	1
CRA	Santa Vitória do Palmar	33° 35’ 37’’	53° 20’ 47’’	1
CRB	Santa Vitória do Palmar	33° 35’ 37’’	53° 20’ 47’’	1
Weedy rice				
AV07	Arroio Grande	32° 19’ 06’’	52° 57’ 01’’	1
AVSC	Cachoeirinha	29° 57’ 05’’	51° 08’ 02’’	0
AV04	Arroio Grande	32° 19’ 30’’	52° 54’ 21’’	0
AV09	Arroio Grande	32° 19’ 41’’	52° 54’ 31’’	1
AV03	Arroio Grande	32° 19’ 20’’	52° 57’ 16’’	0
AVRA	Cachoeira do Sul	30° 05’ 40’’	52° 54’ 12’’	1
AVRB	Cachoeira do Sul	30° 05’ 43’’	52° 54’ 59’’	1

[1]0 =ALS-susceptible or 1 = ALS-resistant

In order to discriminate the ALS resistant (R) and susceptible (S) biotypes, a resistance screening test was carried out from November 2016 to January 2017 in greenhouse conditions located at the Federal University of Pelotas (UFPel). The experimental design was completely randomized, with five replications. The biotypes were seeded in trays of 13.8 L (33 x 54 x 8 cm), filled with autoclaved Albaquaf soil, in rows 10 cm apart with three biotypes per tray. At the three to four leaves stages the barnyardgrass and weedy rice plants were sprayed with imazapyr + imazapic in the label rate of 73.5 + 24.5 g ha^{-1}, respectively. The herbicide was sprayed using a CO_2 backpack sprayer, equipped with four 110.015 air induction flat fan spray nozzle, spaced at 50 cm, with a pressure of 1.0197 kgf cm^{-2}, resulting in an application rate equivalent to 120 L ha^{-1} of spray solution. Weed control was evaluated once at 28 days after herbicide spraying and the biotypes were identified as R or S, adopting a binary scale, where zero (0) means the plant death (susceptible) and one (1) the absence or minimal injuries (resistant).

Selection of a suitable growth medium for barnyardgrass

In order to determine a suitable growth medium for in vitro regeneration of barnyardgrass, an experiment was conducted in September 2016 at the Weed Tissue Culture Laboratory/UFPel. The treatments were arranged in a factorial scheme, with 20 replicates. Factor A consisted of two growth medium: MS 100% (Murashige and Skoog 1962), with sucrose (30 g L^{-1}) and myo-inositol (0.1g L^{-1}); and 50% MS (1/2MS), with sucrose (15 g L^{-1}) and myo-inositol (0.05g L^{-1}). Factor B consisted of the presence and absence of the growth regulator benzylaminopurine (BAP) 1mg L^{-1}. The growth medium pH was adjusted to 5.8 before adding agar (7g L^{-1}), then poured into test tubes (10 mL $tube^{-1}$). The test tubes containing growth medium were sterilized in autoclave (121°C and 1.5 atm) for 20 min and cooled at room temperature until solidification. The 2 cm length barnyardgrass explants (tiller without roots) were collected in greenhouse, disinfected in a laminar air flow chamber, using 70% ethanol and detergent Tween for one minute, then a solution of 1.5% sodium hypochlorite for three minutes was used and finally the explants were rinsing in autoclaved distilled water three times. Additionally, the tillers were immersed in a solution of carboxin + thiram (0.3 + 0.3 g L^{-1}) fungicide for three min, and then placed them in each tube. The test tubes containing barnyardgrass explants were set in a growth chamber under standard conditions (16/8 hours day/night), and a continuous temperature of 24 °C.

At 14 days after the incubation the following measurements were made: shoot and root length (cm), that are the new tissues from explant, and the growth percentage rate using a scale of 0; 50 and 100% (Figure 1). The value of 100% was attributed to the plants that showed maximum growth. Contaminated test tubes with pathogens that had interfered with the plant's growth were not evaluated.

Figure 1. Explants of barnyardgrass according to the scale adopted for growth assessment: 0; 50 and 100% from left to the right test tube.

In vitro imazapyr + imazapic dose-response

The assays were conducted in greenhouse and growth chamber of the tissue growth laboratory. The material used in this study came from the ALS resistance screening, where a susceptible biotype and a resistant biotype were chosen. These biotypes were sown again in trays as described previously, grown until the V9 development stage as a source of tillers to be transferred into the growth medium. The 2-cm length barnyardgrass or weedy rice tillers were disinfected in a laminar air flow chamber, as described previously. The tissue growth medium used was the MS 50% with BAP (1mg L^{-1}) for barnyardgrass (shown the best growth in the experiment above mentioned) and the MS 100%, BAP-free for weedy rice (previously study) (Rey et al. 2009), with the addition of increasing concentrations of the commercial mixture of imazapyr + imazapic herbicide. The herbicide was prepared in stock solution of 100µM of imazapyr + imazapic, which was filtered and aliquots were added to the hot sterilized growth medium and then 10 mL were poured into each test tube.

For an initial verification of the control and growth inhibition of barnyardgrass and weedy rice caused by the herbicide a narrower range of imazapyr + imazapic (0; 0.3; 0.6; 1.2; 2.4 and 4.8µM) was added in the growth medium and incubated the susceptible biotype in a laminar air flow chamber. The parameters of the equation allowed to find the herbicide rate required to achieve 95% of control of the S biotype. Using this information, a second curve for both R and S biotypes was designed. The following concentrations of imazapyr + imazapic were used: 0; 0.225; 0.45; 0.9; 1.8; 3.6; 7.2; 14.4 µM for barnyardgrass and 0; 0.325; 0.65; 1.3; 2.6; 5.2; 10.4; 20.8 µM for weedy rice, using 12 replicates.

After the incubation, the test tubes were placed in a growth chamber, with a photoperiod 16/8 hours of light/dark, and a temperature of 24°C. The control percentage rate and the shoot growth were the parameters assessed, at five and 10 days after inoculation (DAI). Explants that did not show normal development and subsequent necrosis were considered dead. Contaminated test tubes, whose growth was compromised by pathogen were excluded. The length of the shoot explants (cm) was measured from the base to the apex of the tiller and expressed as a percentage relative to the control. The tillers that showed swelling of approximately 1 cm due to the stored reserves, without resprouting, were considered as controlled by the herbicide.

Data analysis

The data collected during the experiment for selecting a suitable growth medium analyzed for normality (Shapiro-Wilk test), homoscedasticity (Hartley test) and subsequently submitted to analysis of variance ($p \leq 0.05$); in case of significance, the means of growth medium and the growth regulator (BAP) were compared by the T-test ($p \leq 0.05$).

The data collected from the first trial with a narrower rate of imazapyr + imazapic for the susceptible biotype were analyzed for normality (Shapiro-Wilk test) and analysis of variance ($p \leq 0.05$) and, in case of significance, were fit in sigmoidal logistic regression. In the second dose response curve, in the case of significance analysis of variance ($p \leq 0.05$), the rates were fit in single exponential regression. The resistance factor (RF) was calculated by the R/S ratio, which corresponds to the division of the C50 (concentration required to obtain 50% control) of the R biotype by the C50 of the S biotype. All statistical tests were carried out with the SAS software (SAS Institute, Cary, NC, USA).

3. Results

Barnyardgrass and weedy rice screening to confirm ALS resistance

Of the 17 barnyardgrass biotypes collected, six survived to the application of the selected herbicide mixture imazapyr + imazapic (73.5 + 24.5 g ha^{-1}) showing only few injuries (Table 1). The recommended herbicide label rate sprayed at the three to four leaf development stage displayed satisfactory control of 65% of barnyardgrass biotypes, that were collected from paddy rice and had previously survived in the last crop season to imazapyr + imazapic (Table 1).

Four of the seven weedy rice biotypes showed few injuries, while three biotypes were susceptible and died due the herbicide exposure (Table 1). The screening allowed to identify six possible resistant biotypes of barnyardgrass and four of weedy rice, which were used in the following assays. The barnyardgrass biotypes CRA and CSB were selected as ALS-resistant and ALS-susceptible biotypes, respectively. Within the weedy rice biotypes, AVRA was selected as ALS-resistant biotype, and AVSC as ALS-susceptible.

Selection of a suitable growth medium for barnyardgrass

According to the result of the analysis of variance ($p \leq 0.05$) there was no interaction between growth medium and BAP for the explant shoot length (data not shown). A simple effect of the plant growth regulator BAP was observed for the explant (tiller without roots) growth and root length (Table 2). In this research, there was no difference of barnyardgrass explants growth between the medium MS 100 % and MS 50%. Then, the medium MS 50% was chosen for further use in the dose-response curve, since the explants had similar development with reduced use of chemicals. A highest barnyardgrass explant growth was verified in the medium with the presence of BAP, with an increased number of new buds (Table 2). The growth regulator at a rate of 1mg L^{-1}, induced the root length, however, these values were statistically similar (Table 2).

Table 2. Percentage of growth and root length of barnyardgrass explants evaluated at 14 days after inoculation in the growth medium.

Treatment	Growth (%)	Root length (cm)
With BAP	65,00 A[1]	0,89 A
Without BAP	45,00 B	0,25 A
VC (%)	48,91	44,20

[1] Means with the same capital letters do not differ from each other according to T test ($p \leq 0.05$). CV: Coefficient of variation

The results found in this experiment allowed to define that the suitable growth medium for barnyardgrass and further used in the dose-response curve experiment was the myo-inositol (0.1g L^{-1}); and 50% MS (1/2MS), with sucrose (15 g L^{-1}) and myo-inositol (0.05g L^{-1}) with BAP at 1mg L^{-1}. The medium did not have any effect on the barnyardgrass explants growth, and for this reason the 50% MS, was chosen to reduce material consume.

In vitro imazapyr + imazapic dose-response for susceptible biotypes

The data obtained for barnyardgrass and weedy rice control was adjusted to a sigmoidal logistic model (Figure 2 A to D). The data of shoot length was fitted an exponential model with three parameters (Figure 2 E and F). At five days after inoculation (DAI), control values around 70% were observed for barnyardgrass in the imazapyr + imazapic rates ranging from 0.6 to 4.8 µM (Figure 2 A). Lower control levels were verified for weedy rice control in the rates of 1.2 and 4.8 µM (Figure 2 B). In the second evaluation, carried out at 10 DAI, the control values did not show the same discrepancy due the better development of the herbicide symptoms (Figure 2 C and D). The control of 100% of barnyardgrass explants were observed at 10 DAI at the rates ranging between 1.2 and 4.8 µM, and the lower rates of 0.3 and 0.6 µM reached the control values of 66.7 and 87.5%, respectively (Figure 2 C). For weedy rice at 10 DAI, a control greater than 90% was observed in rates 1.2; 2.4 and 4.8 µM, while lower rates of 0.3 and 0.6 provide a control ranging from 75 to 90%, respectively (Figure 2 D).

The explants shoot length showed a decreased as the imazapyr + imazapic rates increased (Figure 2 E and F). For barnyardgrass, the imazapyr + imazapic rates ranging from 1.2; 2.4 and 4.8 µM, had a similar effect reducing up to 56% the explants shoot length (Figure 2 E). Even the lower rates of 0.3 and 0.6 µM demonstrated a substantial growth inhibition ranging between 42,5 to 48,75%. Similar results were verified for weedy rice, the explants reduced the shoot length by 70% with the increase of imazapyr + imazapic rates from 1.2 to 4.8 µM (Figure 2 E). The rates 0.3 and 0.6 µM of imazapyr + imazapic also significantly reduced the shoot length, varying from 58 to 61%, respectively.

In vitro imazapyr + imazapic dose-response study

An interaction between both weed biotypes and rates of imazapyr + imazapic herbicide was verified for control (5 and 10 DAI) and explants shoot length (Figure 3). The control data (5 and 10 DAI) was adjusted to an exponential model with three parameters, with R^2 ranging from 0.81 to 0.99 (Figure 3 A to D). The explants shoot length data was adjusted to an exponential model with R^2 ranging from 0.82 to 0.99, depending on biotype and weed species (Figure 3 E and F).

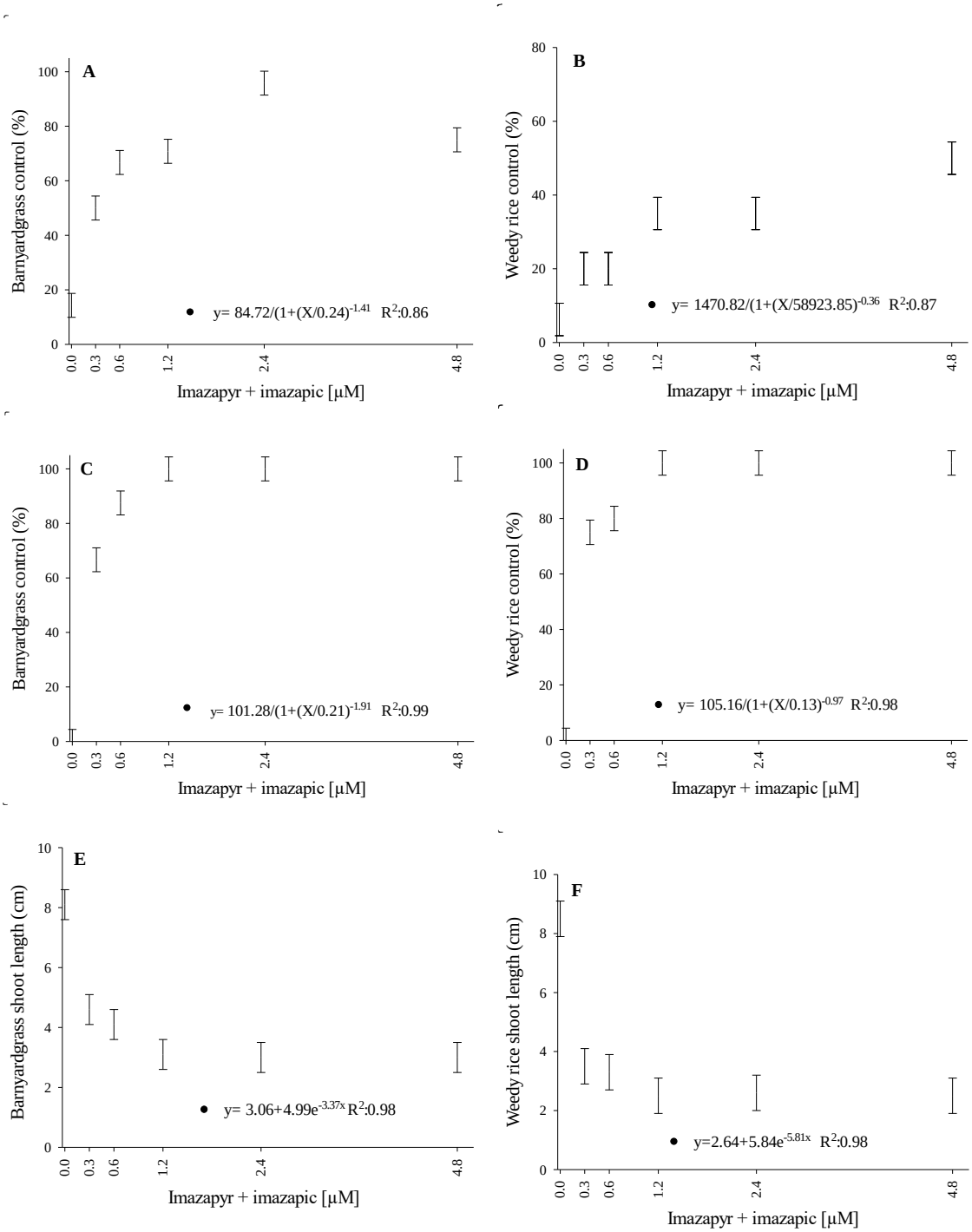

Figure 2. Control (%) of ALS-susceptible barnyardgrass and weedy rice exposed to imazapyr+imazapic rates in the growth medium at 5 (A, B) and 10 (C, D) days after the inoculation (DAI) and shoot length of the explants (E, F) at 10 DAI. Error bars represent 95% confidence interval.

The increasing rates of imazapyr + imazapic in the growth medium, demonstrate that the susceptible and resistant barnyardgrass and weedy rice biotypes has a different response to the herbicide in the evaluation periods. At 5 DAI, the maximum control observed was around 55% for the susceptible barnyardgrass biotype at rates higher than 1.8 µM (Figure 3 A). For the resistant biotype, there was no

differences in control for rates higher than 0.45 µM, with a control rate of about 27%. Due to the symptoms are initials at 5 DAI evaluation, the control rate is low which explains the poor control values for the susceptible biotype, being difficult to differentiate the CRA and CSB biotypes.

For weedy rice explants, a higher control percentage were verified at 5 DAI for the susceptible biotype, being greater than 87% in the rate ranging from 0.65 to 20.8 µM (Figure 3 B). For the resistant biotype, at this evaluation, the dose ranging from 0.32 to 5.2 µM showed control values around 18%, while rates higher than 10.4 µM reached a maximum control of 40%.

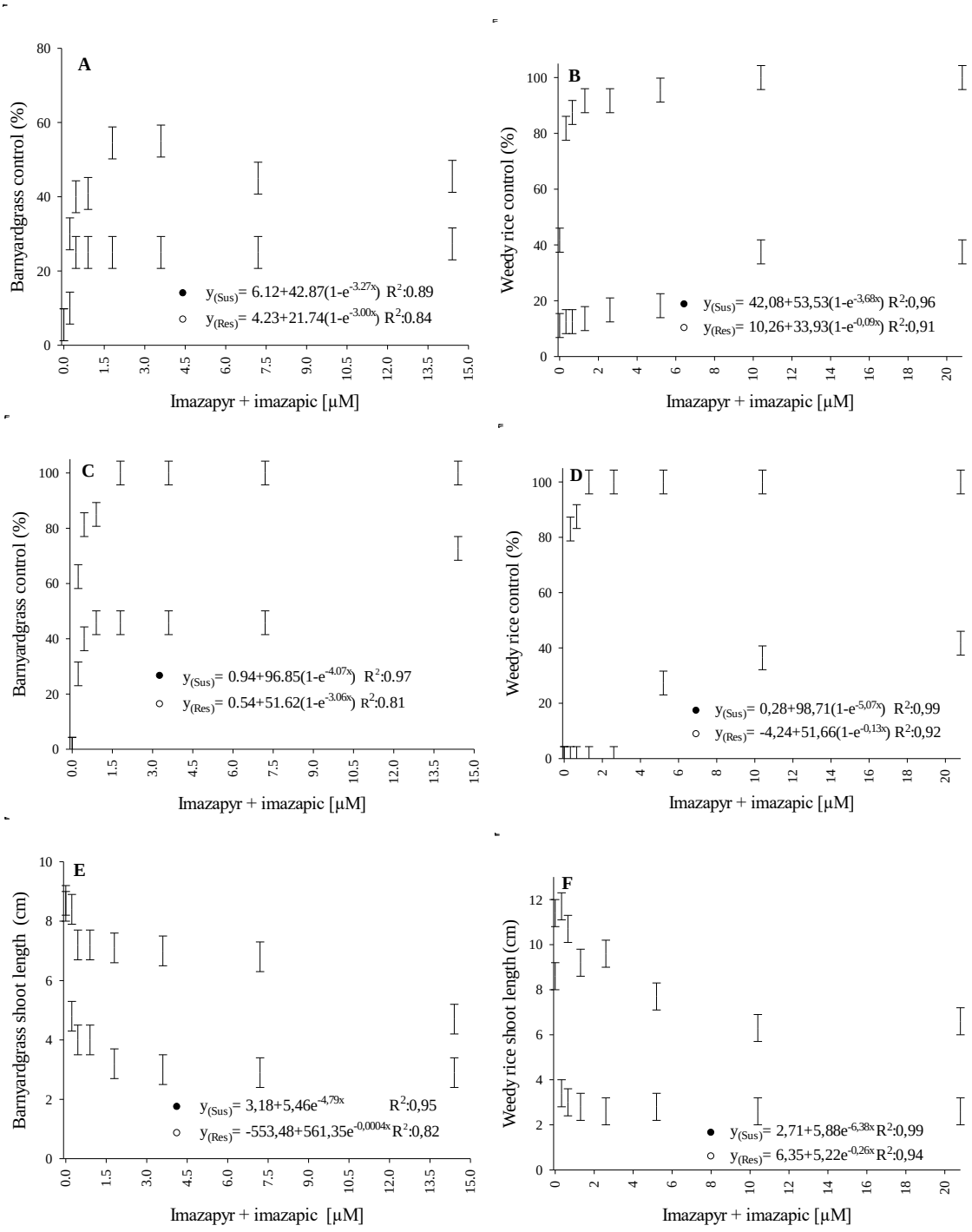

Figure 3. Control (%) of ALS-susceptible and -resistant barnyardgrass and weedy rice biotypes exposed imazapyr + imazapic dose-response curve in the growth medium at 5 (A, B) and 10 (C, D) days after the inoculation (DAI) and shoot length of the explants (E, F) at 10 DAI. Error bars represent 95% confidence interval.

The control of the barnyardgrass susceptible (CSB) biotype at 10 DAI, was much higher than the resistant (CRA), with no differences found for the rates ranging from 1.8 to 14.4 µM, which control were 100% (Figure 3 C). For the resistant biotype, a similar control around 45% was observed in the rates

ranging from 0.45 to 7.2 µM. At the highest rate (14.4 µM) a higher control level (72%) of the resistant biotype was found, differing from the other rates. The herbicide rate required for 50% control of the susceptible biotype was 0.17 µM, while the resistant biotype required 1.04 µM, hence, the RF was 6.0, demonstrating that the CRA biotype was resistant to imazapyr + imazapic herbicide (Table 3).

For weedy rice a 100% control was observed for the susceptible biotype (AVSC) for rates higher than 1.3 µM, while the same rate provided no control of the resistant biotype (AVRA) (Figure 3 D). The resistant biotype did not show any injuries in the rates ranging from 0 to 2.6 µM, where the 2.6 µM correspond for 2x the usual field rate. In addition, the maximum control observed for the resistant biotype were 27, 36 and 42% for rates 5.2; 10.4 and 20.8 µM, respectively, which did not differ from each other. The dose required to control 50% of the resistant biotype was not possible to determine precisely, once the covered rates had a control lower than 50%, as too as the RF estimated higher than 150.5 (Table 3).

Table 3. Herbicide rate required *in vitro* (µM) to promote 50% of control (C50) or reduce by 50% the shoot length (RSL50) with confidence intervals (CI), and resistance factor (RF) of barnyardgrass and weedy rice.

Control at 10 DAI			
Barnyardgrass	C50	CI (p≥0,95)	RF
SUS	0.17	0.00-0.35	-
RES	1.04	0.99-1.09	6.0
Shoot length			
Barnyardgrass	RSL50	CI (p≥0,95)	RF
SUS	0.33	0.15-0.50	-
RES	17.6	17.50-17.70	53.8
Control at 10 DAI			
Weedy rice	C50	CI (p≥0,95)	RF
SUS	0.14	0.07-0.21	-
RES	>20.8	-	>150.5
Shoot length			
Weedy rice	RSL50	CI (p≥0,95)	RF
SUS	0.20	0.16-0.25	-
RES	>20.8	-	>101.5

SUS: susceptible, RES: Resistant.

The evaluation of explant length also allowed to establish differences between resistant and susceptible biotypes (Figure 3E and F). The barnyardgrass explant length demonstrate a decrease according to the increase of herbicide rates, being observed, for the susceptible biotype, a total interruption of explant growth for rates higher than 0.45 µM (Figure 3 E). For the resistant biotype, was not observed the same length inhibition, but there was a gradual decrease according with the rates increasing. However, the highest rate used (14.4 µM) demonstrated a strong inhibition of the explant growth, with a reduction of 45% in shoot length compared to the herbicide-free medium.

The weedy rice shoot length demonstrates a decrease response in a concentration-dependent manner (Figure 3 F). For the susceptible biotype, a very low amount of herbicide was required to achieve a maximum growth inhibition; a lower rate (0.325 µM) demonstrates 75% of shoot growth inhibition. For the resistant biotype, the same growth inhibition was not observed, only a gradual shoot length decreases as rates increased. The imazapyr + imazapic herbicide rates of 0.325 to 0.65 µM, did not demonstrate any reduction in the shoot height with values similar to herbicide-free medium. For this biotype a decrease of 32% in explant length was observed starting from the 5.2 µM rate. However, higher rates did not maximize the explant shoot shortening.

4. Discussion

Barnyardgrass and weedy rice are the most troublesome weeds found in paddy rice of southern Brazil (Fruet et al. 2020). These weeds have increased their presence in rice crop areas recently due the resistance evolution to herbicides (Ulguim et al. 2021). A rapid detection of the resistance in an early season may be an important tool to predict herbicide efficiency before the application and avoid a massive production and dispersion of seeds of these weeds (Kaundun et al. 2011). A rapid detection of the

resistance in an early season may be an important tool to predict herbicide efficiency before the application and avoid a massive production and dispersion of seeds of these weeds (Kaundun et al. 2011). The use of tillers in grasses is a good way to test the herbicide resistance (Schneider et al. 2016), once a dozen of plants collected from the field can provide enough evidence of the real situation of all population. In this work, we seek to use barnyardgrass and weedy rice tillers in tissue culture for the rapid detection of herbicide resistance. No reports for use of tillers and tissue culture techniques for barnyardgrass and weedy rice detection of resistance are available in the literature. This is promising to advances in management and reducing resistance spread for these weed species.

In this study the MS medium independent of the concentration (50 or 100%) did not interfere in the explant growing. Nonetheless a better barnyardgrass explant growth was achieved when BAP was added in the medium. The plant growth regulator BAP act as a synthetic cytokinin that stimulates cell division in plant roots and shoots. The increase in explant growth and root length observed in treatment with BAP is due the activity of cytokinins in promoting cell growth and differentiation leading to the leaf expansion and explant's growth. Cytokinins play an important role in the cell growth, since they control protein synthesis that are directly related to the formation of mitotic spindle fibers (Jelenska et al. 2000).

The phytohormones act in the morphogenesis of explants as a depend manner of the balance between auxin and cytokinins (Christensen et al. 2008). The shoot lengthening generally requires cytokinins, but the amount is dependent of the endogenous concentration produced. Then, in some species it is not necessary addition of cytokinins in the medium (Arab et al. 2014). As a result, the addition of exogenous cytokinins is no longer influential and can even inhibit growth. BAP is generally required in low concentration in the medium ranging from 0.5 to 2.5 mg L^{-1} and higher concentrations of BAP might have an adverse effect on proliferation rate (Arab et al. 2014). For this reason, the medium used for barnyardgrass in the dose response assays were the MS 50% with the addition of BAP at 1mg L^{-1}. For weedy rice the medium used was the MS 100%, BAP-free according previous study (Rey et al. 2009).

The narrower range of imazapyr + imazapic in the growth medium for susceptible biotypes of barnyardgrass and weedy rice provide a useful tool to select rates required to create curves to evaluate resistant and susceptible biotypes. From the parameters of the equation at 10 DAI, the rate needed in the growth medium to provide a control of 95% of the barnyardgrass susceptible biotype was defined as 0.9 µM and 1.3 µM for weedy rice (Figure 2 C and D). In a rapid resistance test (Syngenta RISQ test) with seedling of *Lolium* spp. added in an agar based medium with iodosulfuron + mesosulfuron the death of susceptible seedlings was reached with rates up to 0.1 µM, while the differentiation between dead and survived seedlings was possible at 10 days after treatment (Kaundun et al. 2011). The use of tillers on the agar-based bioassay here proposed also provides a quick herbicide response being possible at 10 DAI. However, the use of seedlings can result in greater control even at very low rates, since plants in the initial development stage are more sensitive to herbicide action, than developed tissues like the tillers used in this study.

The in vitro control evaluation carried out 10 DAI had demonstrated a powerful tool to differentiate between ALS-resistant and susceptible barnyardgrass and weedy rice biotypes. The 10-day interval is faster than 30 or more days usually required to obtain confirmation of resistance in a conventional whole-plant method. The rate of 0.9 µM, discriminated the differential behavior between barnyardgrass biotypes, as it resulted in high control efficiency of the susceptible and very few injuries to the resistant. Similarly, the rate of 1.3 µM discriminated between weedy rice biotypes, as it resulted in high control efficiency of the susceptible (100%) and none control for the resistant. Similarly, the rate of 1.3 µM discriminated between weedy rice biotypes, as it resulted in high control efficiency of the susceptible (100%) and none control for the resistant. However, a larger number of diverse sensitive and resistant populations should be tested to determine the precise discriminating rates of herbicides that reflect the level of resistance in the field.

The low level of control of the weedy rice resistant biotype may be related to the limitation of the doses used in the assay. It is suggested, therefore, that the resistance mechanism of the studied biotype may be related to the site of action of the herbicide (ALS mutation), which is reinforced by high C50 observed and resulting at RF upper than 100 for weedy rice. An increased injury with the highest rates of imazapyr + imazapic for the barnyardgrass resistant biotype might indicates a clue for the resistance mechanism involved this biotype. It is suggested that the resistance mechanism of the biotype is the

enhanced metabolization of the herbicide, which cannot be assert as the unique mechanism involved. Similarly, an agar seedling-based test was adequate to detect ALS and ACCase resistance for pre-determined target and non-target site resistant populations (Kaundun et al. 2011). An increased injury with the highest rates of imazapyr + imazapic for the barnyardgrass resistant biotype might indicates a clue for the resistance mechanism involved this biotype. It is suggested that the resistance mechanism of the biotype is the enhanced metabolization of the herbicide, which cannot be assert as the unique mechanism involved. Similarly, an agar seedling-based test was adequate to detect ALS and ACCase resistance for pre-determined target and non-target site resistant populations (Kaundun et al. 2011).

The movement of the herbicide through the culture medium to the plants during the test has no interference from the barriers and environmental conditions imposed in field conditions, until the herbicide reaches the target. Thus, a resistant plant under normal field conditions may not show resistance during the test in vitro, if the resistance mechanism is related to reduce absorption/translocation of the herbicide, since in the test there is a direct contact with tiller stem and the roots that could grow after inoculation.

Weed resistance to herbicides is an evolutionary process, where its dynamics and impact are dependent on some factors: genetic (number and frequency of resistance genes), biology of plant species (seed production capacity), herbicides (site of action, residual activity) and technical (herbicide rate, spraying technology) (Powles and Yu 2010). As herbicides are selectors of resistant biotypes, the intensity of selection depends on the number of years of use of products with the same site of action in the same area. Early detection of the resistance is important to design new strategies to weed management and avoid crop yield losses.

Most of the current herbicide resistance diagnostic tests are performed after herbicide failure in the field; with the use of whole-plant studies, that require a significant amount of greenhouse space, and might take more than six weeks for resistance confirmation. Petri dish seed and pollen tests are quicker, but mainly detect target site-based resistance and not enhanced metabolism, which is generally developed in later growth stages. The rapid resistance test (Syngenta RISQ test) with seedlings added in an agar-based medium soaked with ALS/ACCase inhibitors had demonstrated a powerful tool to discriminate between susceptible and resistance biotypes in *Lolium* spp. (Kaundun et al. 2011). This method requires seedlings to be inserted in the medium to perform the test, which could be acquired from the seed germination or collecting them from an infested field. However, if seeds are used an adult plant is required to collect them, which could be not available in the early season. On the other hand, seedlings have a very short period in this stage and an adequate amount needed to perform the test could not be available in the field, also remove the soil from seedlings and perform the decontamination prior to insert them in agar-based medium is a labor-dependent task. Here, we describe a simple and quick bioassay using tiller regeneration in vitro assay test and offering the potential for detecting resistance to imazapyr + imazapic in barnyardgrass and weedy rice, prior to herbicide treatment in the field.

5. Conclusions

We have developed a simple, quick, versatile, cost-effective method that can detect resistance to post-emergence imazapyr + imazapic ALS herbicide, for the most troublesome weeds of rice production. The rates of 0.9 and 1.3 µM of imazapyr + imazapic on growth medium from tillers discriminated resistant and susceptible biotypes of barnyardgrass and weedy rice, respectively.

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