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Seed production potential of *Echinochloa colona* exposed to sublethal doses of four commonly-used rice herbicides and high-temperature stress

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Abstract: **Background:** *Echinochloa colona* (junglerice), a troublesome weed in rice, is resistant to 15 herbicide active ingredients. High temperatures are linked to reduction of herbicide efficacy. **Objective:** Evaluate growth and seed production of junglerice, after five generations of recurrent selection with sublethal dose of rice herbicides under heat stress. **Methods:** Junglerice plants previously subjected to recurrent selection with herbicides and heat stress for three cycles were exposed to further iterative cycles of selection with heat stress (45 °C) and sublethal dose of florpyrauxifen-benzyl (FPB, 0.125x), imazethapyr (0.125x), quinclorac (0.25x), and glufosinate (0.25x). Plant injury was evaluated 1 and 3 weeks after treatment. Panicle number; plant height; and dry biomass of shoot , panicles, and seeds were recorded. Seed number plant^{-1} and reproductive effort were estimated. **Results:** The joint effect of heat stress and sublethal dose of herbicides reduced the sensitivity of junglerice to all herbicides tested. Plants treated with FPB and quinclorac produced more shoot biomass and seed number after five generations. Heat-stressed plants treated with FPB produced more seeds than non-stressed plants. Imazethapyr reduced seed production. Heat-stressed junglerice treated with glufosinate produced fewer seeds than non-stressed plants. **Conclusions:** The joint effect of heat stress and sublethal dose of herbicides reduced junglerice sensitivity to the four rice herbicides tested after five generations. Heat stress and sublethal dose of the relatively recent auxinic herbicide florpyrauxifen-benzyl reduced junglerice sensitivity and increased seed production.

Keywords: Abiotic stress; Fecundity; Fitness penalty; Heat stress; Increased fitness; Recurrent selection; Weediness; Weed resistance

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1. Introduction

Echinochloa colona L. (junglerice) is a C_4 grass, mostly autogamous, hexaploid or tetraploid, from tropical and subtropical Asia (Peerzada et al., 2016). Junglerice is one of the most problematic weeds in rice worldwide. Yield losses due to junglerice interference have been reported from 27 to 62% across rice cultivars, with higher losses in direct-seeded rice than in transplanted rice (Fischer et al., 1997). Some traits that make junglerice a successful weed in rice, and in many environments, include crop mimicry, adaptability to flooded and non-flooded crop culture, variable seed dormancy, variable maturation period, high seed production (up to 200,000 seeds per plant), and rapid growth (Tahir, Roma-Burgos, 2021). Herbicides are primary tools for weed control in rice; however, junglerice has evolved resistance to 15 herbicide active ingredients used globally, where 20 out of 26 cases are in rice production fields (Roma-Burgos et al., 2019; Heap, 2023).

Many herbicides are used in rice to control junglerice. These are applied in various combinations at various times in the growing season starting from preplant burndown, pre-emergence, delayed pre-emergence, early post-emergence, pre-flood, post-flood and post-harvest. Auxinic herbicides (group 4 HRAC: quinclorac and florpyrauxifen-benzyl) control junglerice and broadleaf weeds in rice (Epp et al., 2016). Quinclorac can be used pre-plant, pre-emergence, or early post-emergence of rice, providing some residual activity (Grossmann, 1998). Florpyrauxifen-benzyl (FPB) is relatively recently commercialized for rice, with a broader post-emergence spectrum of activity than quinclorac, and is effective on the majority of quinclorac-resistant barnyardgrass (*Echinochloa crus-galli* L.) (Travlos et al., 2020). Glufosinate (group 10 HRAC) is a broad-spectrum post-emergence herbicide used for preplant vegetation burndown in many crops including rice, and in-season nonselective weed control for glufosinate-tolerant crops. Glufosinate controls glyphosate-resistant weed populations such as Palmer amaranth (*Amaranthus palmeri* S. Watson.). Imazethapyr (group 2 HRAC) is a selective herbicide broadly used in Clearfield® rice fields.

Among the various consequences of weed resistance to herbicides is increased weediness such as documented in rigid ryegrass (*Lolium rigidum* Gaud.) (Pedersen et al., 2007), horseweed (*Conyza canadensis*) (Shrestha et al., 2018b), and Palmer amaranth (Vila-Aiub et al., 2014). Glyphosate-resistant junglerice was reported to produce more shoot biomass and seeds per plant than the susceptible genotype (Shrestha et al., 2018a; Mahajan et al., 2020). Conversely, fitness cost has also been reported for glyphosate-resistant goosegrass (*Eleusina indica* (L.) Gaertn.) and imazethapyr-resistant annual bluegrass (*Poa annua* L.) which exhibited 5% and 60% reduction in seed production, respectively (Han et al., 2017; Tseng et al., 2019) but such cases are rarely observed in the field. Quinclorac-resistant junglerice from Arkansas, USA, does not exhibit fitness cost (Penka, 2018) and no fitness penalties have been reported for glufosinate- or FPB-resistant weeds thus far.

Different environmental factors such as high atmospheric temperature and [CO_2] have been reported to reduce efficacy of herbicides. Increases in atmospheric temperature and [CO_2] are consequences of climate change. The best estimate scenario predicts more than 50% chance that the average global temperature will rise by 1.5 °C by 2040, 2 °C by 2050, and up to 2.7 °C by 2100 (Arias et al., 2021). Simultaneous recurrent exposure to heat stress and herbicides across generations reduce the susceptibility of junglerice to the herbicide (Benedetti et al., 2020).

High seed production and multiple resistance to herbicides are some of the main weedy traits of junglerice (Tahir, Roma-Burgos, 2021). Previous research has demonstrated that *Echinochloa glabrescens* Munro ex Hook responds positively to temperature (+19% plant biomass at 37/29 °C day/night), increasing its competitiveness to rice (Alberto et al., 1996). In general, resistance to herbicides does not compromise weed growth or fecundity. A highly glyphosate-resistant junglerice population (resistance index, [RI] = 14) produced 9,300 seeds per plant, similar to the susceptible counterpart (Mahajan et al., 2020). The transcriptome analysis of a junglerice population highly resistant to quinclorac (RI = 32) has shown upregulation of the trehalose pathway, indicative of a non-target site resistance (NTSR) mechanism (Rangani et al., 2022). The enrichment of this pathway suggests increased abiotic stress tolerance of quinclorac-resistant junglerice.

The future scenario for junglerice control portends reduced efficacy of herbicides and increased competitiveness of weed populations. Therefore, understanding the impact of heat stress and recurrent selection with herbicides on the seed production potential and competitive traits of junglerice will help us improve our weed management strategies. We hypothesized that recurrent selection of junglerice with sublethal dose of herbicides under heat stress increases junglerice tolerance to herbicides and weediness. This study aimed to evaluate growth and seed production of junglerice, after three and five generations of recurrent selection with sublethal doses of four commonly used rice herbicides with or without heat stress.

2. Materials and Methods

2.1 Plant materials and growing conditions

Susceptible junglerice seeds were collected in 2011 from a rice field in Prairie County, Arkansas, USA. This field population (Generation 0, G0) was subjected to iterative selection cycle with herbicides and heat stress (Benedetti et al., 2020) until the production of G3 seeds. Tests were conducted in the greenhouse at the Shult Agricultural Research and Extension Center (SAREC), University of Arkansas, Fayetteville, Arkansas, USA. One cycle of selection consisted of submitting V_1 junglerice seedlings to two temperature regimes; 1) heat stress (45/25 °C day/night), and 2) normal (30/25 °C day/night), the heat stress temperature was chosen considering predictions of extreme temperature events in agricultural systems (Arias et al., 2021). Normal temperature was chosen based in the optimal growth temperatures for junglerice (Benedetti et al., 2020; Rao, 2021). These temperature regimes were established using two growth chambers, with a 14-h photoperiod. After 7 d under the respective temperature conditions, plants at stages of V_3 – V_4 were treated with sublethal dose (SLD) of four herbicides in a spray chamber and returned to the same respective growth chambers for another 7 d. The spray chamber was equipped with a compressed air-propelled boom fitted with two flat fan 110.0067 nozzles spaced 50 cm apart and positioned 45 cm above the plant canopy. The sprayer was calibrated to deliver 187 L ha^{-1} at a speed of 1.6 km h^{-1}. After the heat stress period, all plants were placed in the greenhouse and cultured until maturity. The greenhouse average temperature was 32/25 °C (day/night) with a 14-hour daylight supplemented by artificial lights. Seeds of plants from each treatment were collected and used as a new seed line for the next selection cycle (Table 1).

Characterization of injury level on G2 plants revealed minimal, but detectable, reduction in sensitivity to herbicides (Benedetti et al., 2020). Therefore, following the same procedure, G3 plants were subjected to three more cycles of herbicide and heat stress to produce G6 seeds. Pre-test showed reduced sensitivity of G3 plants to some herbicides and prompted us to increase the rate of quinclorac and glufosinate in succeeding selection cycles (Table 1).

2.2 Response of G3 and G5 plants to heat stress and herbicide treatments

Seeds from G3 and G5 populations were germinated in trays filled with commercial potting soil (PRO-MIX BX, Premier-Horticulture Ltd, Quakertown, PA, USA). Individual seedlings were established in 1-Kg pots filled with potting soil and submitted to iterative cycles of heat stress and herbicide, as previously described. G3 seeds were planted on March 7 and harvested on June 13, 2022; and G5 seeds were planted on February 7 and harvested on May 20, 2023. Six weeks after establishment, plants were transferred to bigger pots filled with 2.5 Kg of a 1:3 mixture of potting soil and field soil collected from SAREC, Fayetteville, Arkansas. Pots were placed in dish pans and kept flooded until maturity. Plants were fertilized

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Table 1 - Seed lines of junglerice across iterative cycles of exposure to heat stress and sublethal herbicide dose, Shult Agricultural Research and Extension Center, University of Arkansas, Fayetteville, AR, USA.

Seed line	Temp. regimen, day/night °C	Herbicide			Rate (g a.i. ha^{-1})	
		Active ingredient	Trade name	HRAC group	Iterative selection from G0 to G2[1]	Iterative selection from G3 to G6[1]
1	30/25	nontreated	-	-	-	-
2		florpyrauxifen-benzyl	Loyant	4	3.75	3.75 (0.125x)
3		quinclorac	Facet	4	54	140 (0.25x)
4		glufosinate-ammonium	Liberty 280 SL	10	56	162 (0.25x)
5		imazethapyr	Newpath	2	27	27 (0.125x)
6	45/25	nontreated	-	-	-	-
7		florpyrauxifen-benzyl	Loyant	4	3.75	3.75 (0.125x)
8		quinclorac	Facet	4	54	140 (0.25x)
9		glufosinate-ammonium	Liberty 280 SL	10	56	162 (0.25x)
10		imazethapyr	Newpath	2	27	27 (0.125x)

[1] Field population (G0) and the succeeding two generations (G1 and G2 plants) were selected with sublethal doses of commercial rate of the herbicides (Benedetti et al. 2020). G2 plants were cultured to produce G3 seeds. The selection cycle was continued with G3, G4, and G5 plants to produce G6 seeds. The parentheses represent the herbicide rate used in relation to the recommended commercial rate (1x): 30, 560, 648 and 216 g a.i. ha^{-1} (Loyant, Facet, Liberty, Newpath, respectively).

with 0.75 g L^{-1} of 24-8-16 (N-P-K) water-soluble fertilizer (Miracle-Gro the Scotts Company LLC, Marysville, OH, USA) once every 3 weeks, until panicle initiation. A three-factor (Table 1), completely randomized experiment with six replications was established: 1) temperature (normal: 30 °C and heat stress: 45 °C for 7 d before and after herbicide application), 2) herbicide stress (sublethal dose of four herbicides plus nontreated check), and 3) junglerice generation (G3 and G5). This study consisted of a series of experiments where the core treatments (temperature and herbicide stress) were common for six generations, from G0 to G3 (Benedetti et al., 2020) and herein G3 to G6.

Plant injury was evaluated 1 and 3 weeks after herbicide treatment (WAT) using a scale of 0% (no visible injury) to 100% (dead) compared to the non-treated check (no herbicide). At maturity (90 d after treatment) plant height was measured from the soil surface to the tip of the panicle of the tallest tiller and panicles were counted . Dry biomass of shoots , panicles, and seeds were recorded. Shoots and panicles (without seeds) were oven-dried for 60 h and weighed. The total weight of seeds was recorded, and 500 seeds were weighed to estimate the total number of seeds per plant. The reproductive effort was estimated as the percentage of seed biomass relative to the total above-ground biomass (Equation 1).

$$\text{Reproductive Effort \%} = \frac{(\text{seed biomass})}{(\text{shoot biomass} + \text{panicle biomass} + \text{seed biomass})} * 100 \quad (1)$$

Plant injury means were considered different when the 95% confidence intervals do not overlap. Data for plant height, panicle number, shoot biomass, panicle biomass, seed biomass, seeds per plant and reproductive effort were subjected to analysis of variance ($p < 0.05$) and plots of residual errors were checked for homogeneity of variance. The least significant difference at *p-value* < 0.05 was used for means separation using the Tukey's HSD test. All analyses were performed using JMP® Pro 17.0.0 software.

3. Results and Discussion

3.1 Effect of heat stress in junglerice without herbicide stress

Plants grown under normal conditions (30 °C) without herbicide treatment were taller at G5 than at G3 but had similar shoot biomass (Table 2). Under heat stress (45 °C), plants showed equal height but greater shoot biomass at G5 than at G3. Thus, junglerice plants subjected to heat stress alone produced higher shoot biomass after five generations. The increase in panicle number from G3 to G5 reflects an increase in tiller production across generations, resulting in increased shoot biomass (Table 3). However, increased number of leaves per plant may explain the increase in shoot biomass of heat-stressed junglerice (data not shown). With heat stress alone, panicle biomass declined at G5 compared to G3 (Table 3). The number of panicles per plant was greater at G5 than at G3 of heat-stressed plants. Thus, junglerice produced more, but smaller, panicles per plant after five iterative cycles of heat stress compared to plants grown under normal temperature.

Under normal temperature, without herbicide stress, the number of seeds per plant and seed biomass did not change across generations, as expected (Table 4). On the other hand, heat-stressed G5 plants produced more seeds per plant and seed biomass than G3 plants even without herbicide stress. Therefore, sustained exposure to heat stress alone increases the seed production potential of junglerice, which could be evident in as early as five generations. As mentioned previously, the higher level of seed production is a result of the increased number of panicles per plant rather than bigger panicles. Also, the increase in shoot biomass equates to more photosynthetic tissue supporting higher allocation of assimilates into seeds.

The reproductive effort represents the proportion of aboveground plant biomass allocated to seed, therefore, the pattern here should mirror that of seed production. This is akin to harvest index of crops, except that with weeds, higher reproductive effort indicates higher fecundity, increased weediness, and increased weed management problem. Reduction of reproductive effort is considered as fitness cost (Han et al., 2017) and would be desirable in herbicide-resistant weeds. In the absence of herbicide selection pressure under normal temperature (30 °C), plants showed similar reproductive effort across generations, as expected. However, under heat stress, reproductive effort increased at G5 compared to G3 (Table 5).

3.2 Florpyrauxifen-benzyl (FPB)

G3 plants treated with FPB (3.75 g ai ha^{-1} = 0.125x) under normal temperature (30 °C) incurred 63 and 75% injury at 1 and 3 WAT, respectively (Figure 1). G5 plants incurred 92 and 75% injury at 1 and 3 WAT. Thus, the sensitivity of junglerice to FPB did not change across five generations under normal temperature. The susceptibility of G3 plants to FPB under heat stress (45 °C) was reduced (55%) compared to G3 plants grown under normal temperature. Under heat stress G5 plants showed further reduction in sensitivity to FPB (41.6% at 3 WAT), indicating that junglerice would evolve resistance to FPB faster under high temperature environments.

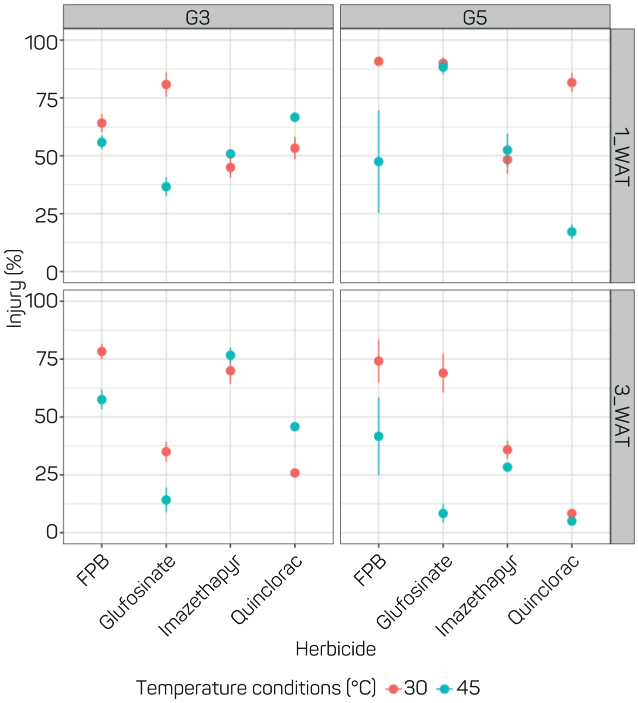

Figure 1 - Junglerice injury at 1 and 3 weeks after treatment (WAT) under two temperatures conditions, 7d prior and 7d after spraying sublethal doses of four herbicides for three and five generations (G3 and G5). Florpyrauxifen-benzyl (FPB: 3.75 g ai ha^{-1} = 0.125x), glufosinate (162 g ai ha^{-1} = 0.25x), imazethapyr (27 g ai ha^{-1} = 0.125x) and quinclorac (140 g ai ha^{-1} = 0.25x). Data points are averages of 6 observations. Bars represent 95% confidence interval

Recurrent selection with FPB resulted in increased shoot biomass at G5 compared to those of G3 plants in either temperature condition (Table 2). Although plants were taller at G5 under normal temperature, plant height did not differ between normal and high temperature. Recurrent selection with FPB resulted in increased panicle biomass at G5 vs. G3 under both temperature conditions (Table 3). This was different from G5 plants exposed to iterative cycles of heat stress without herbicide. After five iterative cycles of sublethal dose of FPB under heat stress, G5 plants had more panicles than G3 plants. Therefore, the joint effect of heat stress and FPB resulted in junglerice with higher seed production potential due to increased number and size of panicles compared to being exposed to herbicide stress alone.

Plants submitted to five iterative cycles of sublethal dose of FPB produced more seeds per plant and greater seed biomass than G3 plants, at both temperature conditions (Table 4). Heat-stressed plants treated with FPB produced more seeds per plant than non-stressed plants as early as the third generation and onward. After five generations of selection with FPB, heat-stressed plants produced more seed biomass than non-stressed plants. Even without heat stress, sublethal dose of FPB increased the seed production potential of junglerice as herbicide-treated G5 plants had higher seed biomass and seed number per plant than G5 plants not exposed to FPB.

Recurrent selection with FPB under normal conditions resulted in reduced reproductive effort of G5 plants compared to G3 plants (Table 5). In contrast, combined heat stress and sublethal dose of FPB increased the reproductive effort of G5 plants compared to G3 plants. This means that the joint effect of heat stress and FPB promotes the allocation of biomass to seed production across generations.

At G2 of FPB-selected seed lines, the fold change of RI compared to that of G0 was 1.7 under heat stress and 1.04 under normal temperature (Benedetti et al., 2020). Data indicated that NTSR mechanisms are driving elevated tolerance in G2 plants exposed to heat stress (Benedetti et al., 2020). Although specific CYP450 enzymes degrading FPB are not yet reported, heat-stressed G2 junglerice exposed to sublethal dose of FPB have shown 4.5-fold upregulation of *CYP72A15* compared to G0 plants at 30 °C (Benedetti et al., 2020). Likewise, FPB-resistant barnyardgrass (RI: 5 to 32) exhibit NTSR mechanisms such as lower herbicide absorption and translocation and reduced bioactivation to the active FPB form (Hwang et al., 2022). Therefore, it is likely that the seed line with reduced herbicide sensitivity generated in this research harbors NTSR mechanisms.

Table 2 - Effect of sublethal doses of herbicides and heat stress in junglerice (*Echinochloa colona*) height and shoot biomass after five cycles of recurrent selection.

Herbicide[1]	Temp.[2] °C	Height (cm)						Shoot biomass (g)					
		G3[3]			G5			G3			G5		
non-treated	30	186.7	ab	B	199.5	a	A	79.7	abc	A	73.1	d	A
	45	201.3	ab	A	212.2	a	A	82.0	ab	B	126.6	a	A
florpyrauxifen-benzyl	30	161.5	cd	B	194.8	ab	A	40.0	e	B	87.0	bcd	A
	45	189.3	b	A	179.2	bc	A	62.0	bcde	B	85.6	bcd	A
quinclorac	30	152.8	d	B	192.5	ab	A	64.1	bcde	B	101.1	bc	A
	45	180.7	bc	B	199.4	a	A	56.0	cde	B	109.8	ab	A
glufosinate	30	169.3	bcd	B	201.0	a	A	53.6	de	B	74.4	d	A
	45	162.3	cd	B	209.4	a	A	93.9	a	A	100.8	abc	A
imazethapyr	30	171.8	bcd	A	139.1	d	B	60.1	bcde	B	82.3	cd	A
	45	174.7	bc	A	166.3	c	A	77.5	abcd	B	97.3	bcd	A
Herbicide (H)[4]		<.0001*						<.0001*					
Temp. conditions (TC)		<.0001*						<.0001*					
Generation (G)		<.0001*						<.0001*					
H*TC		0.0525*						0.0002*					
G*H		<.0001*						0.0003*					
G*TC		0.1817						0.2513					
H*TC*G		<.0001*						<.0001*					

[1] See Table 1 for herbicides rates. [2] Temperature treatment for 7d prior and 7d after herbicide application. [3] Generation 3 and 5, respectively. Means without the same letter are significantly different. Lowercase letters are used to compare means from interaction between herbicide and temperature within the same generation (columns). Uppercase letters are used to compare generation means within the same temperature and the same herbicide (rows). Means are separated based on Tukey's honest significant difference (HSD) test (α = 5%). [4] ANOVA at 95% of confidence level.

Table 3 - Effect of sublethal doses of herbicides and heat stress in junglerice (*Echinochloa colona*) panicles per plant and panicle biomass after five cycles of recurrent selection.

Herbicide[1]	Temp.[2] °C	Panicles plant^{-1}						Panicle biomass (g)					
		G3[3]			G5			G3			G5		
non-treated	30	92.7	abc	A	115.2	bc	A	6.6	a	A	3.1	bc	B
	45	68.3	c	B	118.7	bc	A	5.7	a	A	4.0	bc	B
florpyrauxifen-benzyl	30	78.7	bc	A	87.7	c	A	1.6	b	B	3.0	bc	A
	45	85.0	abc	B	145.3	b	A	2.6	b	B	4.2	bc	A
quinclorac	30	94.5	abc	A	99.7	c	A	1.9	b	B	3.4	bc	A
	45	68.3	c	B	78.4	c	A	2.6	b	B	7.6	a	A
glufosinate	30	115.3	ab	A	105.0	bc	A	3.1	b	A	2.7	bc	A
	45	94.5	abc	A	207.3	a	A	3.0	b	A	2.4	bc	A
imazethapyr	30	121.3	ab	A	89.7	c	B	3.3	b	A	2.5	bc	A
	45	125.0	a	A	80.3	c	B	3.1	b	A	2.4	c	A
Herbicide (H)[4]		0.0143*						<.0001*					
Temp. conditions (TC)		0.1081						0.0004*					
Generation (G)		<.0001*						0.3275					
H*TC		<.0001*						<.0001*					
G*H		<.0001*						<.0001*					
G*TC		<.0001*						0.0021*					
H*TC*G		<.0001*						0.0046*					

[1] See Table 1 for herbicides rates. [2] Temperature treatment for 7d prior and 7d after herbicide application. [3] Generation 3 and 5, respectively. Means without the same letter are significantly different. Lowercase letters are used to compare means from interaction between herbicide and temperature within the same generation (columns). Uppercase letters are used to compare generation means within the same temperature and the same herbicide (rows). Means are separated based on Tukey's honest significant difference (HSD) test (α = 5%). [4] ANOVA at 95% of confidence level.

Table 4 - Effect of sublethal doses of herbicides and heat stress in junglerice (*Echinochloa colona*) seeds per plant and seed biomass after five cycles of recurrent selection.

Herbicide[1]	Temp.[2] °C	Seeds plant^{-1}						Seed biomass (g)					
		G3[3]			G5			G3			G5		
non-treated	30	19,357	abcd	A	18,899	de	A	27.9	abc	A	27.5	cd	A
	45	17,509	bcd	B	33,600	a	A	22.2	bc	B	43.8	a	A
florpyrauxifen-benzyl	30	15,457	d	B	22,454	cd	A	23.1	bc	B	30.1	bc	A
	45	17,328	cd	B	29,908	ab	A	23.3	bc	B	42.2	a	A
quinclorac	30	15,386	d	B	25,385	bc	A	22.4	bc	B	37.1	ab	A
	45	14,628	d	B	29,141	ab	A	21.5	c	B	41.6	a	A
glufosinate	30	24,295	a	A	27,206	bc	A	34.8	a	A	38.4	ab	A
	45	20,487	abcd	A	16,804	de	A	30.1	ab	A	24.3	cd	B
imazethapyr	30	24,249	ab	A	16,190	e	B	31.2	ab	A	20.5	d	B
	45	21,553	abc	A	14,480	e	B	24.6	bc	A	18.8	d	B
Herbicide (H)[4]		0.0097*						<.0001*					
Temp. conditions (TC)		0.2772						0.0038*					
Generation (G)		<.0001*						<.0001*					
H*TC		<.0001*						0.2765					
G*H		<.0001*						<.0001*					
G*TC		0.0007*						<.0001*					
H*TC*G		<.0001*						<.0001*					

[1]See Table 1 for herbicides rates. [2]Temperature treatment for 7d prior and 7d after herbicide application. [3]Generation 3 and 5, respectively. Means without the same letter are significantly different. Lowercase letters are used to compare means from interaction between herbicide and temperature within the same generation (columns). Uppercase letters are used to compare generation means within the same temperature and the same herbicide (rows). Means are separated based on Tukey's honest significant difference (HSD) test (α = 5%). [4]ANOVA at 95% of confidence level.

Table 5 - Effect of sublethal doses of herbicides and heat stress in junglerice (*Echinochloa colona*) reproductive effort after five cycles of recurrent selection.

Herbicide[1]	Temp.[2] Conditions	Reproductive effort (%)					
		G3[3]			G5		
non-treated	30	24.6	cde	A	27.6	abc	A
	45	20.4	e	B	25.3	cd	A
florpyrauxifen-benzyl	30	36.0	ab	A	25.7	bc	B
	45	26.5	cde	B	31.5	ab	A
quinclorac	30	25.5	cde	A	26.9	bc	A
	45	26.9	cd	A	26.3	bc	A
glufosinate	30	38.1	a	A	33.5	a	B
	45	23.8	de	A	19.2	de	B
imazethapyr	30	30.6	bc	A	19.5	de	B
	45	23.5	de	A	16.0	e	B
Herbicide (H)[4]		<.0001*					
Temp. conditions (TC)		<.0001*					
Generation (G)		<.0001*					
H*TC		<.0001*					
G*H		<.0001*					
G*TC		0.0011*					
H*TC*G		0.0002*					

[1]See Table 1 for herbicides rates. [2]Temperature treatment for 7d prior and 7d after herbicide application. [3]Generation 3 and 5, respectively. Means without the same letter are significantly different. Lowercase letters are used to compare means from interaction between herbicide and temperature within the same generation (columns). Uppercase letters are used to compare generation means within the same temperature and the same herbicide (rows). Means are separated based on Tukey's honest significant difference (HSD) test (α = 5%). [4]ANOVA at 95% of confidence level.

The rate of resistance evolution to FPB has not been assessed before using the recurrent selection approach. The fact that junglerice becomes less sensitive to FPB after five generations of selection under heat stress and produces more seeds per plant is a threat to rice production because this portends more severe infestations, larger weed seedbank, and increased occurrence and dispersal of resistant individuals harboring NTSR mechanism as the global temperature continues to rise. The mechanisms governing the increase in seed production of heat-stressed plants are yet to be investigated. We hypothesize that expression of genes related to oxidative stress protection is the cause of this phenomenon. This warrants further investigation.

3.3 Quinclorac

Quinclorac (140 g ai ha^{-1} = 0.25x) caused 25% injury to G3 plants at 3 WAT under normal conditions (30 °C) (Figure 1). At G5, junglerice became even less sensitive to quinclorac causing only 8% injury at 3 WAT. G5 plants initially showed high injury (81%, 1 WAT) but recovered quickly (8% injury at 3 WAT), showing faster recovery than G3 plants. Under heat stress (45 °C), G3 plants treated with quinclorac showed 46% injury at 3 WAT, which was higher than the injury observed on G3 plants under normal temperature. Heat-stressed G5 plants showed higher tolerance to quinclorac (5% injury at 3 WAT) than heat-stressed G3 plants and higher tolerance than G5 plants grown under normal temperature. Thus, junglerice sensitivity to quinclorac declined significantly after five generations of recurrent selection regardless of temperature.

Similar to FPB, recurrent selection with sublethal dose of quinclorac resulted in taller plants and greater shoot biomass at G5 than at G3, regardless of temperature conditions (Table 2). Like the effect of recurrent selection with FPB, selection with quinclorac resulted in greater panicle biomass at G5 than at G3 regardless of the temperature conditions (Table 3). The panicle number was the same between G5 and G3 plants under normal temperature but increased at G5 after iterative exposure to quinclorac and heat stress. Additionally, the panicle biomass was greater at G5 heat-stressed plants than non-stressed plants. Thus, heat-stressed junglerice treated with quinclorac resulted in higher seed production potential due to increased size of panicles compared to junglerice exposed to herbicide stress alone. Recurrent selection of junglerice with quinclorac for five cycles increased the number of seeds per plant and seed biomass compared to G3, with or without heat stress (Table 4). Similar to FPB, quinclorac promoted the seed production potential of junglerice without heat stress. The reproductive effort of plants submitted to sublethal dose of quinclorac did not change across generations and temperature conditions (Table 5).

Prior experiments on G2 plants from the quinclorac seed line revealed 1.39-fold change in RI under heat stress and 1.01 under normal temperature compared to G0 (Benedetti et al., 2020). Under heat stress, *HSP15* (heat shock protein) was upregulated 8.37-fold, indicating its role in mitigating the effects of heat stress. Studies have shown that resistance of *Echinochloa phyllopogon* (RI: 6 to 17) and barnyardgrass (RI: 14 to 35) to quinclorac is caused by inhibition of ethylene biosynthesis and the degradation of hydrogen cyanide (HCN) by increased beta-cyanoalanine synthase (β-CAS) activity (Yasuor et al., 2012; Zia et al., 2020). Accessions of junglerice resistant to quinclorac (RI: >32) from Arkansas, USA, harbor different mechanisms of resistance since the resistant and susceptible accessions exhibited the same activity of this enzyme (0.3194 and 0.3192 M Na_2S) (Rouse et al., 2019). Transcriptome analysis of this population revealed upregulation of the trehalose pathway, which is known to contribute to abiotic stress tolerance and may also contribute to quinclorac resistance (Rangani et al., 2022). The reduction in sensitivity of G5 plants to quinclorac may be caused by a combination of different mechanisms. Although seed production did not differ under normal and high temperature, the values suggest a tendency to increase seed production under heat stress.

3.4 Imazethapyr

The manifestation of imazethapyr (27 g ai ha^{-1} = 0.125x) activity on junglerice was slower than that of the other herbicides tested (Figure 1). The optimum effect of imazethapyr was manifested at 3 WAT with G3 plants showing 70% vs. 45% injury at 1 WAT under normal conditions (30 °C). Two additional cycles of selection resulted in significantly less sensitivity to imazethapyr. G5 plants also showed some level of recovery from injury at 3 WAT in contrast to G3 plants, which did not recover. The sensitivity of G3 plants to imazethapyr did not change under heat stress (45 °C) compared to plants growing in normal temperature. Likewise, the sensitivity of heat-stressed G5 plants to imazethapyr was similar to that of G5 plants growing in normal conditions. It was interesting to see G5 plants showing the ability to recover to some extent, which was not observed among G3 plants.

Plants treated with imazethapyr produced more shoot biomass at G5 compared to G3 regardless of temperature (Table 2). After five generations of exposure to sublethal dose of imazethapyr, the plants became shorter under normal temperature whereas the plant height of heat-stressed plants remained the same as that of earlier generations. Imazethapyr reduced the panicle number of G5 plants, with or without heat stress, while panicle biomass did not change across generations (Table 3). Contrary to the effect of auxinic herbicides, plants submitted to five recurrent selection cycles with imazethapyr produced less seeds per plant and less seed biomass than G3 plants

(Table 4). This can be attributed to the decrease in panicle number of G5 plants compared to G3 plants. Imazethapyr-treated G5 plants showed reduced reproductive effort with or without heat stress.

The faster recovery of junglerice to ALS inhibitor (imazethapyr) after five generations of recurrent selection indicates increased ability to repair damages and regenerate, regardless of temperature. Early generation (G2) of this seed line showed 1.58 fold-change in RI under heat stress but no change under normal temperature compared to G0 (Benedetti et al., 2020). Barnyardgrass submitted to two recurrent selection cycles with imazethapyr did not show elevation in resistance factor compared to G0 (Rigon et al., 2023). Our experiment indicates reduction in sensitivity to imazethapyr at G5, but bioassays still need to be conducted to determine the fold-change in sensitivity.

Plants submitted to recurrent selection with imazethapyr produced more shoot biomass after five cycles, but less seed yield; hence, the reproductive effort was less at G5 than at G3. This is indicative of fitness cost. Reduction in fitness as a consequence of the evolution of a resistant phenotype has also been reported with other herbicides. For example, a 5% reduction in reproductive effort has been associated with target-site resistance to glyphosate in goosegrass (LD_{50}-based resistance factor > 182) (Han et al., 2017). It is known that ALS mutation is the most predominant mechanism endowing resistance to ALS inhibitors in *Echinochloa* spp. (or other weed species) and some of these mutations may carry fitness cost (Panozzo et al., 2021). One example is the 60% reduction in grain yield of ALS-resistant annual bluegrass harboring L574W mutation (Tseng et al., 2019). At this point, G5 plants are not yet resistant to the full dose of imazethapyr. Considering that all resistance-conferring ALS mutations endow resistance to the full dose, reduced sensitivity to imazethapyr is most likely due to NTSR mechanisms rather than target site mutations. Such mechanisms could imbue fitness cost.

3.5 Glufosinate

Glufosinate (162 g ai ha^{-1} = 0.25x) caused high injury to G3 plants (80 and 35% at 1 and 3 WAT, respectively) under normal temperature (Figure 1). The injury level remained high with G5 plants, indicating that unlike the auxinic herbicides and imazethapyr, junglerice sensitivity to glufosinate did not decline after five generations of iterative selection. On the other hand, G3 plants incurred less injury from glufosinate treatment under heat stress (36 and 14% at 1 and 3 WAT, respectively) than G3 plants growing under normal temperature. G5 plants incurred high injury initially (88%, 1 WAT) but recovered almost completely (8% injury) when placed under heat stress for two weeks flanking the time of herbicide application. This data suggests that although the sensitivity of junglerice to glufosinate did not decline across five generations, G5 plants treated with glufosinate under heat stress recovered faster from injury than plants growing under normal conditions.

Five iterative cycles of exposure to sublethal dose of glufosinate resulted in taller plants regardless of temperature (Table 2). Heat-stressed G5 plants treated with glufosinate produced greater shoot biomass. This increase in shoot biomass can be attributed to more tiller production since heat-stressed G5 plants had almost double the panicle number (indicating increased tillering) compared to plants in normal temperature (Table 3).

G5 plants treated with glufosinate had similar number of panicles and panicle biomass as G3 plants. Heat-stressed G5 plants showed increased number of panicles compared to plants under normal temperature. Since panicle biomass was not different between temperature conditions, we can deduce that sublethal dose of glufosinate with heat stress increased panicle number but reduced the panicle size. Repeated exposure to glufosinate did not affect junglerice seed production across generations (Table 4). Heat-stressed plants submitted to five cycles of recurrent selection with glufosinate had less seed biomass than plants under normal temperature. Iterative exposure to glufosinate resulted in larger plants with less seeds after five generations. Hence G5 plants subjected to glufosinate selection pressure, had lower reproductive effort than G3 plants (Table 5). The reproductive effort was also lower for heat-stressed plants than for non-stressed plants.

Unlike the effects of auxinic herbicides and imazethapyr, junglerice sensitivity to glufosinate did not decline after five generations of iterative selection under normal temperature, but G5 plants selected with glufosinate under heat stress recovered faster from injury. Characterization of the early generation of these seed lines (G2) showed a reduction in sensitivity to glufosinate under heat stress (RI: 2.04) but not under normal temperature (RI: 1.1) compared to G0 (Benedetti et al., 2020). The fact that heat-stressed junglerice recovered almost fully at 3 WAT suggests faster resistance evolution to glufosinate under elevated temperatures. To date, goosegrass, annual bluegrass and annual ryegrass (*Lolium perenne* ssp *multiflorum*. Lam.) have been reported resistant to glufosinate, but not junglerice (Heap, 2023). Thus, our data serve as an early warning that rising global temperature could escalate the evolution of resistance to glufosinate similar to what has been reported with other herbicide-weed interactions.

Glufosinate is an inhibitor of glutamine synthetase (GS), and multiple isoforms have been identified (Takano and Dayan, 2020). Among the isoforms, *GS1-1*, *GS1-2* (cytosolic), and *GS2* (plastidic) have drawn the attention of researchers studying the resistance evolution to glufosinate (Noguera et al., 2022; Zhang et al., 2022). Mutations in *GS1-1* (S-59-G) have been reported to endow resistance to glufosinate in goosegrass (Zhang et al., 2022). Metabolic differences of glufosinate suggest NTSR mechanism in annual ryegrass (Brunharo et al., 2019). In Palmer amaranth, the overexpression and increased copies of *GS2* endows glufosinate resistance (Noguera et al., 2022). Further research should focus on understanding the joint effect of temperature in resistance evolution to glufosinate and ways to mitigate this undesirable consequence.

Considering that heat-stressed plants submitted to glufosinate for five cycles recovered faster from injury, but had lower reproductive effort, this indicates that the allocation of more resources to support faster repair and regrowth had sacrificed the allocation of photosynthates to the development of reproductive organs. Increased biomass production was also reported among junglerice populations resistant to glyphosate compared to the susceptible counterpart under normal temperature (66%) and under salt stress (50%) (Shrestha et al., 2018a). However, glyphosate-resistant junglerice yielded similar to the susceptible counterpart (Mahajan et al., 2020). No cases of fitness cost have been documented among the few glufosinate-resistant weed species thus far, either because resistance does not come with fitness penalty, or fitness effects have not been evaluated. This subject also warrants further investigation.

In summary, three out of four herbicides (FPB, quinclorac and imazethapyr) commonly used in rice caused reduction of junglerice sensitivity after five cycles of recurrent selection under heat stress. This conforms with results of related research conducted earlier in our research facility (Refatti et al., 2019). Reduction in junglerice control by cyhalofop has been reported under high temperature (38/26 °C, day/night) (Refatti et al., 2019). Likewise as was done in this recent study, characterization of the earlier generation showed that heat-stressed G2 plants of the current seed lines already exhibited small, but detectable, reduction in sensitivity to herbicides compared to plants under normal temperature (Benedetti et al., 2020). Further studies should focus on exploring the causes of the joint effect of heat stress to recurrent selection of herbicides in junglerice control and weediness.

4. Conclusions

Recurrent exposure of junglerice to sublethal doses of herbicides under heat stress (45 °C) accelerates resistance evolution. Recurrent selection with FPB under heat stress increases weediness through increased seed production. Increased tolerance to sublethal doses of quinclorac and imazethapyr is apparent after five generations, but fecundity is not affected by heat stress. On the other hand, recurrent selection with sublethal doses of glufosinate for five generations does not reduce sensitivity but improves the recovery rate from injury. Simultaneous exposure to glufosinate selection pressure and heat stress reduces RE, but not with glufosinate alone. This study portrays the consequences of heat and herbicide and selection pressure to four commonly used herbicides in rice production. This information informs us on future scenarios for rice weed management.

Authors' contributions

All authors read and agreed to the published version of the manuscript. NRB and JCV: Conceptualization of the manuscript and development of the methodology. JCV: data collection and curation. JCV: data analysis. NRB and JCV: data interpretation. NRB: funding acquisition and resources. NBR: project administration. NRB: supervision. NRB and JCV: writing the original draft of the manuscript. NRB and JCV: writing, review and editing.

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