Differential effects of glyphosate on germination and chlorophyll in *Zea Mays* plants

Hajar El Hendaoui[1], Khadija Boudarga[2], Mostafa Kandil[3]

Département de Biologie, Equipe des sciences anthropogénétiques et biotechnologie, Faculté des Sciences El Jadida Maroc

Abstract— *We studied the glyphosate effect on the germination and chlorophyll content of two corn varieties. The first variety is local "beldi" not improved and the second is a variety selected and imported. Our analysis showed that the imported variety is more tolerant than local. The latter would have been affected by the direct effects of glyphosate by oxidative stress manifesting itself by a strong inhibition of germination and an increased degradation of chlorophyll. However, the imported variety behaved as capable of degrading glyphosate to aminomethylphosphonic acid resulting in improved herbicide resistance.*

Keywords— Chlorophyll, Germination, Glyphosate, Maize, Morocco.

I. INTRODUCTION

Since the introduction of glyphosate-resistant plants, products derived from this herbicide have become the most widely used in the world. Glyphosate was considered one of the least toxic herbicides (Williams and al., 2000, Cerdeira and Duke 2006). Aminomethylphosphonic acid (AMPA) is the main degradation product of glyphosate by microorganisms and has been detected in soil and water (Cerdeira and Duke.2006). The Glyphosate effects on the plants physiological processes have been recently examined (Gomes and al., 2014) with deleterious effect on photosynthesis and germination. These effects are thought to be related to oxidative stress factors and it is known that the decrease in photochemical efficiency is associated with a decrease in chlorophyll content (Mateos-Naranjo and al 2009, Zobiole and al.2011). Disruption of mineral nutrient uptake (Mn and Fe) by glyphosate has been shown to affect the biosynthesis of chlorophyll (Cakmak et al., 2009). However, studies on the effects of glyphosate and mineral nutrition are contradictory (Bailey and al, 2002, Rosolem and al., 2010). Reddy et al. 2004, showed that AMPA is responsible for the deleterious effects observed on the biosynthesis of chlorophyll in soybeans. It is important to note that the decrease in chlorophyll content has been observed in plants that do not degrade glyphosate to AMPA (Mateos-Naranjo et al 2009, Huang et al., 2012).Oxidative stress was observed in plants exposed to glyphosate (Ahsan et al 2008), as in many other plants exposed to herbicides. It is widely accepted that modulation of Reactive oxygen species (ROS) metabolism may affect plant physiology by inducing chlorophyll degradation and functional losses of chloroplasts (Pitzschke et al., 2006).

Due to the importance of maize crops in Morocco, its use in animal and human nutrition and the existence of an unimproved local "beldi" variety used by Doukkala's farmers, we studied the effects of glyphosate on germination and chlorophyll content in the local variety "beldi" and comparing it with another imported "paulina".

II. MATERIAL AND METHOD

2.1 Plants

To test the effect of glyphosate on germination and chlorophyll, we chose two varieties of maize. A first local variety produced and consumed by farmers in the Doukkala semi-arid region (Morocco), whose grains have an obvious heterogeneity in size and morphology. A second variety imported and marketed under the name paulina, it is characterized by size and morphology grains of uniform.

2.2 Experimentation

2.2.1 Germination

Germination studied by setting up the following experimental setup: for each variety, we made two replicates for the control and four for two glyphosate doses. One repetition corresponds to a basin containing 80 grains, making a total of 1600 grains for both varieties. We arranged the basins randomly. We used Glyphosate Herbicide marketed as 36% of isopropylamine salt (360g / l).

In order to highlight the effect of glyphosate on germination, we used two doses. The first is the one recommended by the providers of the product namely 42 mM, and the second is twice the recommended dose (84 mM). The control was treated

with distilled water. The germination was carried out with a photoperiod of 16 h and a temperature of 20 ° C. After two consecutive washes with distilled water, the grains were disinfected with 2% sodium hypochlorite and rinced in disttilled water.

For the control, we moistened the wattman paper with distilled water. However for the rest we used two glyphosate solutions at 42 mM and 84 mM for humidification. After depositing the grains, we covered them with another layer of wattman paper. Each day, distilled water and glyphosate solutions are sprayed onto the wattman paper for each basin to maintain moisture favorable for germination. The number of sprouted grains is noted after 4, 8 and 12 days of germination. The germination rate per basin is calculated according to the formula:

$$\% = \frac{\text{number of sprouted grains}}{\text{total grains}} * 100$$

2.2.2 Chlorophyll

2.2.2.1 Plant culture protocol

After seed germination, they are transplanted into 13 cm diameter pots filled with soil and peat (3/4 and 1/4). The test consisted of sixty pots, 30 pots for each variety distributed as follows: 10 for control, 10 for 42 mM and 10 others for 84 mM. The distribution of the pots in the culture chamber is random in two blocks. Each block contains 30 plants for both varieties, corresponding to five replicates for each treatment (control, 42 mM and 84 mM). The plants are watered with distilled water at a photoperiod of 16h and at 20 ° C. At the three-leaf stage, 40 plants of both varieties were treated with both doses of glyphosate. After 8 and 15 days of treatment, we performed chlorophyll assays.

2.2.2.2 Chlorophyll Determination

Chlorophyll extraction was performed according to LEE's protocol slightly modified (1981). 0.5 g of leaves are crushed in a mortar with 10 ml of pure ethanol and centrifuged at 10000 g for 10 min. To achieve complete exhaustion 2 additional ethanol extractions are performed. The pooled supernatant (30ml) frequently having a turbidity, a 10ml aliquot is subjected to a new centrifugation. The optical density of the clear extract is then measured at 649 nm and at 665 nm with a Unikon spectrophotometer.

III. RESULTS AND DISCUSSION

3.1 Germination

Germination begins with the release of the radicle, and then the coleoptile develops, adventitious roots then appears. Results for the effects of glyphosate on the germinability of corn kernels are given by variety in Tables 1 and 2.

3.1.1 Unimproved local variety "Beldi"

After 4 days of germination, we found highly significant differences (p = 0.007) between the three treatments. In fact, with distilled water, germination rate is 58.12% whereas for 42 mM and 84 mM doses the rates are low 29.37% and 20% respectively. Glyphosate treatment caused a reduction in germination. In addition, those for 8 and 12 day durations, the differences are not significant between treatments. This could be related to an early adaptation of germinating grains to glyphosate.

TABLE 1
GERMINATION RATES AND EFFECTS OF GLYPHOSATE DOSES ON THE LOCAL VARIETY

Duration Germination	Treatment glyphosate	Average number of sprouted kernels.	(% Rate)	Standard error	F-Test
4 Days	Distilled water	46.5	(58,12)	3,5	11.016 ** p=0.007
	42 Mm	23,5	(29,37)	3 ,57	
	84 Mm	16	(20,00)	4,30	
8 Days	Distilled water	58,5	(73,12)	16,5	3,729 NS p=0.079
	42 Mm	39,5	(49,37)	5,33	
	84 Mm	30,75	(38,43)	2,56	
12 Days	Distilled water	58,5	(73,12)	16,5	3,027 NS (p=0.113)
	42 Mm	39,5	(49,37)	5,33	
	84 Mm	33	(41,25)	3,26	

3.1.2 Variety imported "paulina":

It is noted that for the three durations, the differences between the treatments are not significant. The paulina variety showed early tolerance from the first days with germination rates comparable to those of control. We can deduce this variety is tolerant to glyphosate.

TABLE 2
GERMINATION RATES AND EFFECTS OF GLYPHOSATE DOSES ON VARIETY IMPORTED "PAULINA"

Duration Germination	Treatment glyphosate	Average number of sprouted kernels.	(% Rate)	Standard error	F
4 Days	Distilled water	73,5	(91,87)	1,5	3,953 NS (p=0,071)
	42 Mm	74,00	(92,50)	1,68	
	84 Mm	54,25	(67,80)	8,04	
8 Days	Distilled water	76,50	(95,62)	0,5	2,204 NS (p=0.181)
	42 Mm	74,00	(92,50)	2,27	
	84 Mm	64,00	(80,00)	5,75	
12 Days	Distilled water	76,50	(95,62)	0.50	2,425 ns (p=0,158)
	42 Mm	74 ,50	(93,12)	1,84	
	84 Mm	64,00	(80,00)	5,75	

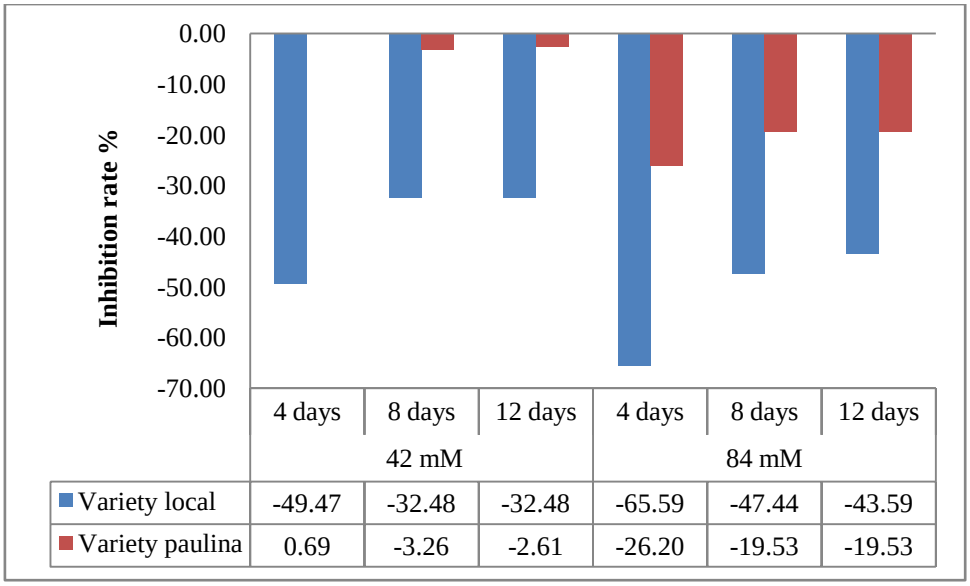
3.1.3 Comparisons between varieties:

We compare the germination rates of two varieties; the results are shown in Table 3.

TABLE 3
COMPARISONS OF GERMINATION RATES BETWEEN THE TWO VARIETIES BY THE T-TEST.

Duration Germination	Treatment glyphosate	Variety	Average number of sprouted kernels.	(% Rate)	Standard error	F
4 Days	Distilled water	Local	46,50	(58,12)	3,50	7,091* (P=0.019)
		Paulina	73,50	(91,87)	1.50	
	42 Mm	Local	23,50	(29,37)	3,57	12,793*** (p=0)
		Paulina	74,00	(92,50)	1,68	
	84 Mm	Local	16,00	(20,00)	4,301	4,193** (p=0.006)
		Paulina	54,25	(67,80)	8,04	
8 Days	Distilled water	Local	58,57	(73,12)	16,50	1,090 NS (p=0,389)
		paulina	76,50	(95,62)	0,50	
	42 Mm	Local	39,50	(49,37)	5,33	5,953*** (p=0.001)
		Paulina	74,00	(92,50)	2,27	
	84 Mm	Local	30,75	(38,43)	2,56	5,275** (p=0.002)
		Paulina	64,00	(80,00)	5,75	
12 Days	Distilled water	Local	58,50	(73,12)	16,50	1,090 NS (p=0.389)
		Paulina	76,50	(95,62)	0,50	
	42 Mm	Local	39,50	(49,37)	5,33	6,203*** (p=0,001)
		Paulina	74 ,50	(93,12)	1,84	
	84 Mm	Local	33,00	(41,25)	3,26	4,682** (p=0,003)
		Paulina	64,00	(80,00)	5,75	

We calculated the inhibition rate of germination in both varieties; these are the differences between the germination levels of the control and those found for the two doses, expressed as a percentage. The inhibition rates are shown in Figure 1. The highest inhibition percentages are those recorded in the local variety. The mean rate of inhibition, all doses and duration combined, is -45.17% and -11.74% in the local variety and Paulina respectively. In both varieties, whatever the dose, the effect inhibition decreases with duration, this would be in relation with a gradual adaptation to glyphosate.



	42 mM			84 mM		
	4 days	8 days	12 days	4 days	8 days	12 days
Variety local	-49.47	-32.48	-32.48	-65.59	-47.44	-43.59
Variety paulina	0.69	-3.26	-2.61	-26.20	-19.53	-19.53

FIGURE 1: Inhibition of germination according to doses and duration

Studies of the glyphosate effects on seed germination are not many. (Blackburn and Boutin, 2003) and most often contradictory, with deleterious effects (Morash and Freedman, 1989, Shuma and al. 1995, Gomes and al. 2014, 2016) or no effects observed (Egley and al., 1978, Piotrowicz-Cieslak and al., 2010).

When the effects are deleterious there is a significant decrease in the germination rate, which we have highlighted in our study. This reduction in germination rate would be accompanied by a reduction in the respiration rate and an accumulation of hydrogen peroxide. Glyphosate has been shown to induce oxidative stress in plants by the accumulation of Reactive oxygen species (ROS) (Gomes and al., 2016). ROS have important role in plants; they participate in the depletion of seed endosperm, the mobilization of reserves, protection against pathogens, and as signaling molecules (Gomes and Garcia, 2013). ROS are involved in germinating seeds; ROS elimination systems play a central role in the germination process (El-Maarouf-Bouteau, Bailly, 2008). If these systems are no longer able to control oxidation rates, there is a disruption of germination. ROS damage cellular components such as proteins, lipids and DNA (Gill and Tuteja, 2010). Also, it has been shown that glyphosate can interfere with the electron transport chain mitochondrial in *Lemna minor* leaves, resulting in ROS accumulation (Gomes and Juneau, 2016). In addition, Lopez-Brana and al. 1984 showed on the mitochondria isolated of corn that protein synthesis as well as mitochondrial respiration is reduced. If the herbicide could have similar toxic effects on the respiratory metabolism of seeds, it could affect seed germination by inducing oxidative stress.

3.2 Chlorophyll:

Chlorophyll content and comparisons between the two varieties as a function of treatment duration and dose are given in Table 4.

3.2.1 Control

After 8 days of treatment with distilled water, comparisons of chlorophyll levels between the two varieties showed highly significant differences ($p = 0.004$). In the local Moroccan variety, we found a rate of 69.89 µg / ml whereas in the Paulina variety we recorded 39.12 µg / ml. The photosynthetic power of the local variety is far superior to that of the imported variety paulina. After 15 days, we find that the statistical differences are very highly significant ($p = 0.001$). In addition, we note that the local variety has a chlorophyll level of 88.30 µg / ml which is double compared to that found in the paulina variety 44.21 µg/ml.

3.2.2 Glyphosate treatment: 42mM and 84mM

After 8 days of treatment the differences between the two varieties are significant at 42 mM. In fact, the average chlorophyll content in paulina variety is 33.08 µg / ml which is greater than that of the local variety 17.93 µg / ml. It noted that paulina has a very good tolerance to glyphosate at this concentration. After 15 days of treatment, the differences between the two varieties are significant at 42 mM and content chlorophyll is very low in the local variety. However, at 84Mm the leaves in both variety became necrotic and we could not measure chlorophyll levels.

TABLE 4
COMPARISONS OF CHLOROPHYLL CONTENT BETWEEN THE TWO VARIETIES BY THE T-TEST

Exposure Duration	Treatment	Variety	Chlorophyll µg/ml	Standard error	Test t
8 Days	Distilled water	Paulina	39,12	0,09	6,162** (p=0.004)
		Locale	69,90	4,99	
	42 Mm	Paulina	33,08	4,37	3,921* (p=0,017)
		Locale	17,93	3,32	
	84 Mm	Paulina	31,25	7,29	1,324 N.S, (p=0.256)
		Locale	19,33	5,52	
15 Days	Distilled water	Paulina	44,21	4,00	8,979***, (p=0.001)
		Locale	88,30	2,84	
	42 mM	Paulina	12,64	1,36	3,020* (p=0,039)
		Locale	5,545	1,92	
	84 mM	Paulina	Not determined		
		Locale	Not determined		

We estimate the reduction rates in the amount of chlorophyll by dose and exposure duration expressed as a percentage. The results are shown in Figure 2. The reduction rates, all doses and durations combined, are 77.35% and 34.68% for the local variety and paulina respectively.

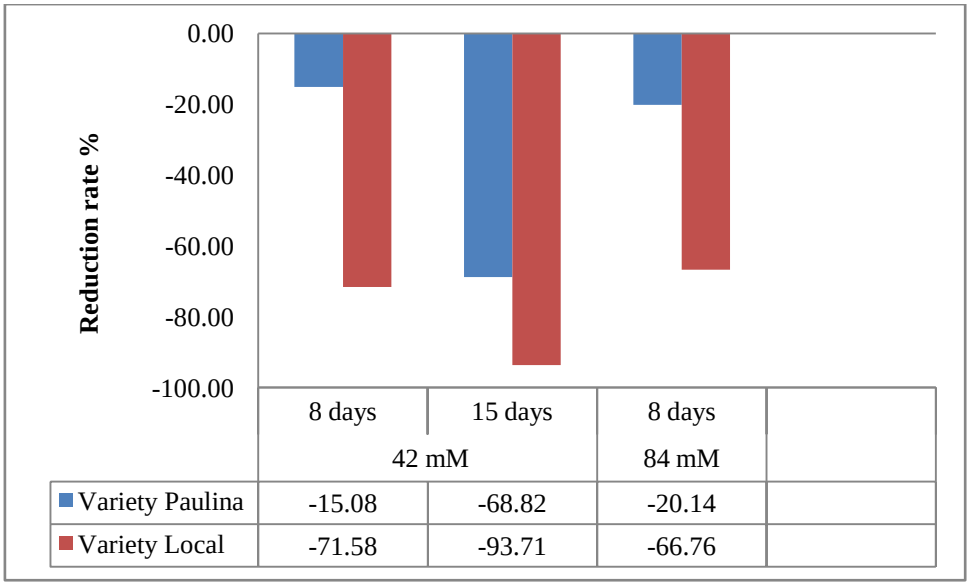


FIGURE 2: Reduction of chlorophyll content as a function of duration and concentration of the herbicide %

The results of the present study show that glyphosate has phytotoxic effects and causes a decrease in chlorophyll content with significant differences between the two varieties. Indeed, the same observations have been made in various species (Ahsan and al., 2008, Miteva et al., 2010, Maroli et al., 2015), involving glyphosate in the disturbance of plant photochemistry. Chlorophyll was significantly reduced after exposure to glyphosate. According to Gomes et al. 2015, these reductions are due to biosynthesis inhibition and/or increased chlorophyll degradation, glyphosate induces degradation of chlorophyll by increasing lipid peroxidation and a significant H2O2 accumulation in treated plants. An increase in H2O2 accumulation and lipid peroxidation following exposure to glyphosate was also observed in rice (Ahsan et al., 2008) and pea (Miteva et al., 2010). Once accumulated, H2O2 will react with subcellular components that may cause enhanced oxidative damage. In contrast, the willow plants treated with AMPA, a degradation product of glyphosate, show an inhibition of the chlorophyll biosynthesis. AMPA directly affects the biosynthesis of chlorophyll by competing with glycine, and / or in the active site of δ-aminolevulinic acid (ALA) synthetase, and deprives plants of the substrates necessary for the biosynthetic pathway chlorophyll (Serra, and al 2013). The induction of peroxide accumulation after treatment with AMPA does not appear to be sufficient to induce oxidative stress; this has been observed in *Arabidopsis thaliana*, indicating that the effects of AMPA are

not due to damage oxidative agents (Serra, and al 2013). Gomes and al. 2015 observed the highest levels of H2O2 in glyphosate-treated leaves compared to AMPA-treated plants.

In this context, the chlorophyll reduction in the local variety is twice that of the imported variety, which is explained by a difference in the degradation of glyphosate in both varieties. Given the difference in behavior of the two varieties, we propose that the paulina variety degrades glyphosate to AMPA, which inhibits the synthesis of chlorophyll, whereas the local variety undergoes the direct oxidative effects of glyphosate which degrade chlorophyll.

IV. CONCLUSION

We have demonstrated that the responses of two varieties of Zea Mays with respect to glyphosate are different. The local variety "Beldi" suffered more damage than the imported variety with a sharp reduction in germination and increased degradation of chlorophyll, which would correspond to the direct oxidative effects of glyphosate. However, in the imported variety, the germination capacity and chlorophyll content are less affected, suggesting that this variety could degrade the herbicide to AMPA and tolerate the direct oxidative effects of glyphosate. Additional studies to confirm our results are the determination of content pheophytin of glyphosate treated plants. Pheophytin is one of the degradation products of chlorophyll. Indeed, the pheophytin / chlorophyll ratios inform us if there is an effect on the biosynthesis of chlorophyll and / or on its degradation. In addition, an increase in H2O2 accumulation and lipid peroxidation after exposure to glyphosate should be verified. Finally, to better understand the process involved in the metabolism and accumulation of H2O2, we will study the activities of antioxidant enzymes (SOD, CAT and APX).

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