Research article

Naphthalic anhydride decreases persistence of alachlor and atrazine and elevates tolerance of maize

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ABSTRACT

The present study aimed at alleviating the impacts of alachlor (Ala) or atrazine (Atr) on maize growth by seed-dressing with naphthalic anhydride (NA, 0.4% w/w by seed weight). The dressed and undressed seeds were germinated for 10 days and treated with Ala or Atr at 3.2 or 1.8 kg ha^{-1}, respectively, then the herbicide residues were determined in shoots one day after treatment. Atr residues were higher than Ala and remained in the same level for the 2nd day then retracted consistently up to 12 days. Meanwhile, GSH and GST were significantly induced while growth parameters were reduced; the effect of Atr was higher than Ala. Nonetheless, ABA, phenolics and anthocyanins as well as PAL, TAL and CI were increased but IAA was decreased coincidently with enhanced IAA-O and peroxidase. The immediate detection of the herbicide residues could conclude that growth reduction as elucidated from the decreased IAA concomitant with elevating ABA, phenolics and anthocyanin contents and enzyme activities are consequences of the herbicide persistence. The drop of IAA was preceded by the stimulation of IAA-O and peroxidase while the increased phenolics and anthocyanins followed PAL, TAL and CI stimulation confirming the regulatory roles of these enzymes. The application of NA greatly lowered the herbicide residues concurrently with ameliorations in growth parameters, GSH, GST, and maintained the balancing of secondary metabolites and plant growth regulators. Lowering Ala and Atr residues by NA in synchronization with enhanced GSH and GST could conclude that NA encouraged the detoxification of the herbicide. Moreover, the balances of IAA, ABA, phenolics and anthocyanins were mostly maintained in normal levels concomitantly with growth ameliorations suggesting that phytohormones and secondary metabolites are involved in the elevation of maize tolerance to Ala and Atr.

1. Introduction

Ala and Atr herbicides are widely used for the control of annual grasses and broadleaf weeds in maize fields. Ala is an α-chloroacetanilide herbicide inhibits some metabolic processes during initial seed germination and further development such as protein synthesis (Sharp, 1988). Atr [2-chloro-4-ethylamino-6-isopropylamino-1,3,5-triazine] belongs to S-triazine herbicides blocks the electron flow between the photosystems (Dodge, 1994). Herbicides drastically influence all aspects of primary and secondary metabolism in crop plants when given to control weeds. The susceptibility of crops to these herbicides depends on their persistence and/or detoxification. As a whole, the plant resistance to herbicides was attributed to less sensitivity of the target site and/or increased herbicide degradation (Nemat Alla et al., 2007; Huang et al., 2019). Several metabolic processes in plants are slowed down due to the inhibitory effects of herbicides on plant metabolism. In general, a number of enzymatic systems are able to detoxify herbicides in plants. These include GST that adds the tripeptide GSH to herbicides and cytP450 that hydroxylates herbicides. O-Glycosyltransferases may further detoxify hydroxylated herbicides. Glycosylation and glutathione conjugation of herbicides are signals for extrusion of compounds from the cytoplasm, either into the vacuole or into the apoplast. Like other α-chloroacetanilide and S-triazine herbicides, Ala and Atr are mainly detoxified in higher plants by their conversion into GSH derivatives by the catalytic activity of GST. Anderson and Davis (2004) stated that GST utilizes GSH to play an important role in plant defense mechanism. On the other hand, Pyon et al. (2004) indicated that one of the paraquat-resistant mechanisms in *Erigeron canadensis* might be related to detoxicative enzymes and GSH content. Moreover, Zabalza et al. (2007) detected that the enhancement of GSH in imazethapyr-treated pea roots can be related to the detected increase of glutathione reductase activity.

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In addition, the growth of plants could be influenced by the alteration in the endogenous plant growth regulators balance. Some changes in plant growth regulators were recorded in response to abiotic stress (Zaric et al., 1983; Mackay et al., 1990). Beside the influence on plant growth regulators, herbicides may affect secondary metabolism (Lydon and Duke, 1989); their products are controlled by some enzymes including PAL (EC 4.3.1.1), TAL (EC 4.3.1) and CI (EC 5.5.1.5). These enzymes are considered as switching for the production of terpenoids, isoflavonoids, anthocyanin, etc. that act as precursors of signaling molecules essential for defense system against stresses (Gottstein et al., 1991).

Herbicide tolerance is a highly desirable trait in crops that can be explained by effectors of stress adaptation that mediate overcoming of herbicide impacts. To cope with harsh conditions, plants developed endogenous defense mechanisms; however, these mechanisms are often not enough in the susceptible species. The protection of plants from herbicide phytotoxicity could be promoted by exogenous supporters to mitigate the herbicide effects (Jang et al., 2019) or by safeners such as NA through increasing herbicide detoxification (Hatzios, 2000). Generally, safeners are compounds that are utilized to protect crops from herbicidal injury (Hatzios, 2000; Deng and Hatzios, 2003; Nemat Alla and Hassan, 2008). So, exogenous supporters are needed to protect the plant from herbicides for withstanding the stress conditions. NA enhanced the conjugation of several herbicides with GSH (Deng and Hatzios, 2003) or hydroxylation by cytP450 (Dalazen et al., 2018). Safener-induced protection in cereals is associated with increased GSH content and increased expression of herbicide detoxifying enzymes, including GSTs (DeRidder et al., 2002). The expression of GST genes was induced in wheat by herbicide safeners (Xu et al., 2002). Moreover, Gao et al. (2019) reported that safeners increased GSH and the expression levels of herbicide detoxifying enzymes, including GST, catalase, and POD in maize. In addition, NA enhanced acetolactate synthase and acetyl CoA carboxylase activities, and protected photosystem II electron transport rate (Hwang et al., 1997; Krenchinski et al., 2019). With these effects of NA, other routes of mode of action might be included such as maintaining the balances of plant growth regulators and secondary metabolites. However, the use of herbicides must be minimal due to their contamination to the environment and also the intensive use of herbicides will select weed populations with resistance to herbicides. So, the present study aimed at alleviating the impacts of Ala and Atr on maize growth and elevating herbicide tolerance by using NA. Specifically, the maintaining of plant growth regulators and secondary metabolism in addition to decreasing the herbicide persistence were checked to be involved in the action of NA for mitigating the herbicide toxicity. For this purpose, maize grains were dressed with NA (0.4% w/w by seed weight), germinated for 10 days then treated with Ala or Atr at 3.2 or 1.8 kg ha^{-1}, respectively. Plant growth, GSH and the herbicide residues were tested as well as some the growth regulators (ABA and IAA), the secondary metabolites (phenolics and anthocyanins) and the related enzymes (GST, IAA-O, POD, PAL, TAL and CI).

2. Materials and methods

2.1. Plant material and growth conditions

A part of maize grains (*Zea mays* L.) was dressed with NA (0.4% w/w by seed weight) by shaking the seeds in a flask and another part was left as control. Fifteen seeds were planted in quartz sand, to avoid the interference with herbicide degradation, (pre-washed with HCl) approximately 2 cm-depth in plastic pots (40 cm × 20 cm × 10 cm) and spaced 5 cm apart in 5 cm adjacent rows. The pots of each experiment were distributed randomly into trays in greenhouse at 30 ± 5/20 ± 5 °C, day/night temperature with a 14-h photoperiod and watered from overhead to the soil surface as required. When seedlings were 10-day-old, the control pots were divided into three groups; one to continue as control (Cont) and the other two groups for treatment with Ala (the trade name is Lasso and the manufacturer is Monsanto) or Atr (the trade name

is Aatrex and the manufacturer is Syngenta) at the rate equivalent to the recommended field doses (3.2 or 1.8 kg ai ha^{-1}, respectively). The dressed NA pots were divided into two groups; one for Ala (Ala + NA) and the other for Atr (Atr + NA) treatments at the same doses. To apply the required dose of the herbicide, the rate per hectare was calculated according to the surface area of the pots then the herbicide was mixed in 4 L water and Tween-20 (0.25%, v/v) was added as a surfactant, then applied to all pots (each pot received 200 mL) with a laboratory mechanical sprayer with one nozzle twice, in one direction and crosswise. The shoots were harvested at the time of herbicide application (zero time) and at 2, 5, 8, and 12 days after the herbicide treatment, washed with copious amounts of water to get rid of any adsorbed herbicide and blotted dry with paper towels and used for the subsequent analyses, a part was weighed for recording the fresh weights then dried in oven at 80 °C for 2 days for dry weight measurements. Determination of herbicide residues was performed after one day of treatment and continued for the other intervals.

The overall area of the pots that were used in the study was 8.0 m^2 for all treatments. Fifty pots (40 × 20 × 10 cm) were used for the experiment, which is repeated twice (100 pots for 2 independent experiments). The area of pots for each treatment was represented by 1.6 m^2 (0.4 m × 0.2 m dimensions × 10 replications × 2 repetitions).

2.2. Determination of herbicide residues

Residues of Ala and Atr were determined according to Marucchini et al. (1988). Fresh samples (5 g) were homogenized with methanol, evaporated and residues were taken up with chloroform/methanol (95/5 v/v) to a chromatographic column (Ø = 2.5 cm) containing 10 g acidic aluminum oxide. The eluent was evaporated and rinsed with 0.5 mL chloroform and then transferred to a chromatographic column (Ø = 1 cm) containing 5 g florisil and eluted with chloroform. After evaporation of the eluent, the residues were dissolved in 2 mL benzene and 2 μL were injected onto GC with carrier gas (N$_2$ at 30 mL min^{-1}), detector gases (air and H$_2$ at 80 and 7 mL min^{-1}, respectively), oven and injector temperatures (200 and 240 °C, respectively).

2.3. Determination of GSH, phenolics and anthocyanin

GSH was extracted from fresh samples (5 g) in 5% trichloroacetic acid containing 10 mM EDTA and centrifed at 12000 × *g* for 15 min (Anderson and Gronwald, 1991). GSH was assayed in phosphate buffer (100 mM pH 6.8) containing 10 mM EDTA, 1 mM CDNB and 1.0 U equine GST. After incubation at 35 °C for 30 min, the absorbance at 340 nm was recorded before commencing the reaction and after the reaction had run to completion. Phenolics were extracted from fresh samples (5 g) with 75% aqueous ethanol, centrifuged and assayed using Folin-Ciocalteau reagent then absorbance was measured at 765 nm (Singleton and Rossi, 1965). Anthocyanin was extracted from fresh samples (5 g) in acidic methanol (HCl, 1%), centrifuged for 20 min at 5000 × *g* and quantitated by measuring the difference in absorbency at 525 nm and 585 nm (Hoagland, 1980).

2.4. Determination of ABA and IAA

ABA and IAA were extracted, purified and determined according to Majcherczyk et al. (1986). Fresh samples (5 g) were homogenized with acetonitrile containing 2,6-di-tertbutyl-p-cresol and partitioned with chloroform, dried and re-dissolved in methanol. ABA and IAA fractions were separated by preparative HPLC, collected and evaporated to dryness. Methanol (500 μL) was added and 10 μL were injected onto analytical HPLC with the mobile phase methanol containing 0.05% acetic acid/water containing 0.05% acetic acid (20/80 v/v) in linear gradient over 30 min to final (70/30 v/v). The flow rate was 1 mL min^{-1} at 255 nm and the retention time was 27.4 and 22.5 min for ABA and IAA, respectively.

2.5. Assay of enzyme activities

GST was extracted in Tris-HCl (100 mM, pH 7.5) containing 2 mM EDTA, 14 mM β-mercaptoethanol and 7.5% polyvinyl polypyrrolidone. After centrifugation at 15000 × g for 15 min, ammonium sulfate was added to 80% saturation (Dixon et al., 1995). GST was assayed in phosphate buffer (100 mM, pH 6.5) containing 5 mM GSH and 1 mM CDNB, incubated at 35 °C, the reaction was stopped by HCl and absorbance was measured at 340 nm. The enzyme activity was calculated by the extinction coefficient E = 9.6 mM^{-1} cm^{-1} (Askelof et al., 1975). IAA-O was assayed by following the residual IAA left after reaction in phosphate buffer (50 mM pH 6.5) containing 0.1 mg IAA (in 0.5 mM CaCl$_2$) and 0.1 mM 2,4-dichlorophenol (Malik and Singh, 1980). After incubation in the dark at 30 °C for 30 min, the reaction mixture was mixed with equal volume of Salkowski reagent and incubated at 30 °C, and then absorbance at 530 nm was recorded. POD was assayed in citrate buffer (50 mM, pH 5.6) containing 0.1 mg O-dianisidine and H$_2$O$_2$ and the rate of increase in absorbance at 430 nm was recorded every 30 s (Mahanta et al., 1993). PAL, TAL and CI were extracted in Tris-HCl (50 mM, pH 8.4) containing 15 mM β-mercaptoethanol and centrifuged at 24000 × g for 20 min. PAL and TAL activities were assayed in Tris-HCl (0.5 mM, pH 8) containing either 6 μmol of L-phenylalanine (for PAL) or 5.5 μmol of L-tyrosine for (TAL) (Beaudoin-Egan and Thorpe, 1985). After the incubation at 30 °C, the reactions were stopped by the addition of 50 μL of 5 N HCl and the absorbance was read for PAL and TAL at 290 and 333 nm, respectively. CI activity was assayed in Tris-HCl (0.5 mM, pH 8) containing 10 μg of chalcone (dissolved in 10 μL of ethylene glycol monomethyl ether), incubated at 37 °C and the decrease in absorptivity was recorded at 375 nm (Hahlbrock et al., 1970).

2.6. Statistical analysis

The design was completely randomized and repeated twice comprises 100 pots (5 set treatments) × (10 replications) × (2 repetitions). The mean values (±SD) were applied (n = 6). The full data were first subjected to ANOVA followed thereafter by LSD at 5% level.

3. Results

Residues of Ala and Atr herbicides were determined in shoots of 10-day-old maize seedlings after one day of treatment up to the following 12 days. Figure 1 represents that both herbicides were persistent in shoots of treated seedlings; the persistence of Atr was higher than that of Ala. Anyway, the persistence of Ala and Atr was highest during the first 2 days representing about 97 % on the 2nd day relative to values detected on the first day. Thereafter, the persistence of both herbicides slightly retracted consistently to become 79, 55 and 40% on the 5th, 8th and 12th day, respectively for Ala and to become 82, 66 and 45%, respectively for Atr. Nevertheless, seed-dressing with NA resulted in significant withdrawn of the detected residues of both herbicides. On the 2nd day from treatment, the persistence of Ala was significantly retracted by NA. The magnitude of Ala residues represented about 58% of the initial value that detected on the 1st day and represented 59% relative to residues detected in samples without seed-dressing on the 2nd day. Thereafter, Ala persistence decreased to 37, 24 and 11%, respectively on the 5th, 8th and 12th day relative to the initial values. In samples treated with only Ala, the residues retracted to 59, 47, 44 and 28% on the 2nd, 5th, 8th and 12th day, respectively. Similarly, NA caused significant drop in the persistence of Atr, it decreased the residues to 61% on the 2nd day relative to the initial value and to 63% relative to that detected on the 2nd day. From the 5th day onward, Atr residues were decreased to 48, 27 and 19%, respectively relative to the initial values and 58, 40 and 43%, respectively in relation to the respective samples treated with only Atr.

Meanwhile, GSH content reached a maximum at day 2 due to all the tested treatments, then from this point onwards the contents began to decrease. Anyway, GSH levels in samples treated with Ala or Atr were
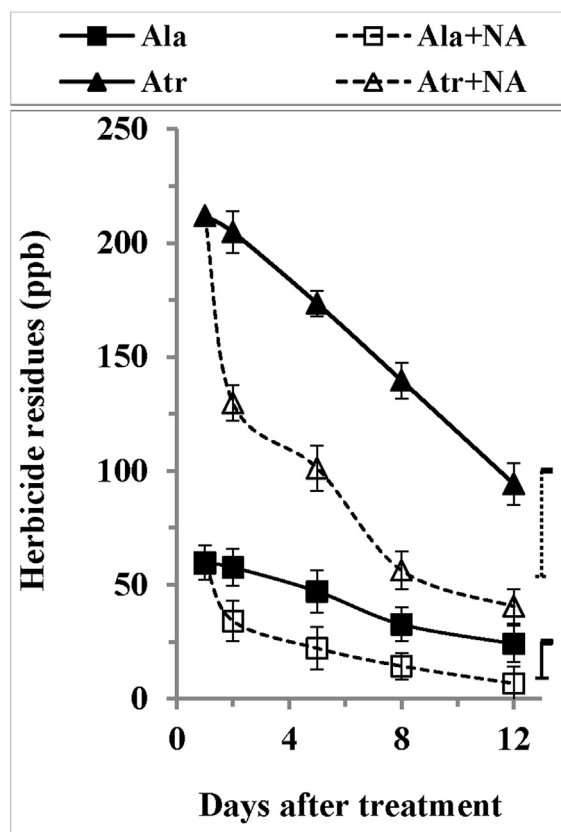

Figure 1. Influence of naphthalic anhydride (NA) on herbicide residues in shoots of 10-day-old maize seedlings treated with alachlor (Ala) and atrazine (Atr) during the following 12 days. Values are means ± SD (n = 6). Vertical bars (entire for Ala, dotted for Atr) represent LSD at $p < 0.05$.

higher than the control during the first 5 or 8 days, respectively, thereafter GSH contents became close to control (Figure 2). Seed-dressing with NA led to further increases in these levels up to the 8th day, then a retraction was set in at the 12th day. Similarly, treatment with Ala or Atr provoked significant inductions in the activity of GST up to the 8th day from treatment; the magnitude of induction by Ala was higher than by Atr (Figure 2). The application of NA led to great stimulation in the enzyme activity up to the 8th day from treatment; thereafter the change in the enzyme activity became non-significant as compared to control.

Regarding the changes in fresh weight, dry weight and water content of maize shoots of 10-day old seedlings during the following 12 days after treatment with Ala or Atr herbicides without or with seed-dressing with in NA, Figure 3 demonstrates that fresh and dry weights were significantly decreased by Ala during the first 5 days and by Atr during the first 8 days, thereafter both herbicides had non-significant effects. Water content was significantly decreased during the first 2 and 8 days by Ala and Atr, respectively. However, maize seed-dressing with NA tended to counterbalance the herbicide effects on maize growth; the significant effects induced by both herbicides became non-significant after the 2nd day of treatments.

The contents of IAA were significantly depressed by Ala and Atr during the first 5 and 8 days, respectively, thereafter they became comparable to that of control, the magnitude of depression was more pronounced by Atr than Ala (Figure 4). On the contrary, ABA contents were significantly increased by Ala during the first 5 days and by Atr during the first 8 days, then both herbicides became with non-significant effects. The treatment with NA counterbalanced both the drop in IAA levels and the increases in ABA induced by Ala or Atr such that these levels reached comparable values to control from the 2nd or the 5th day, respectively.
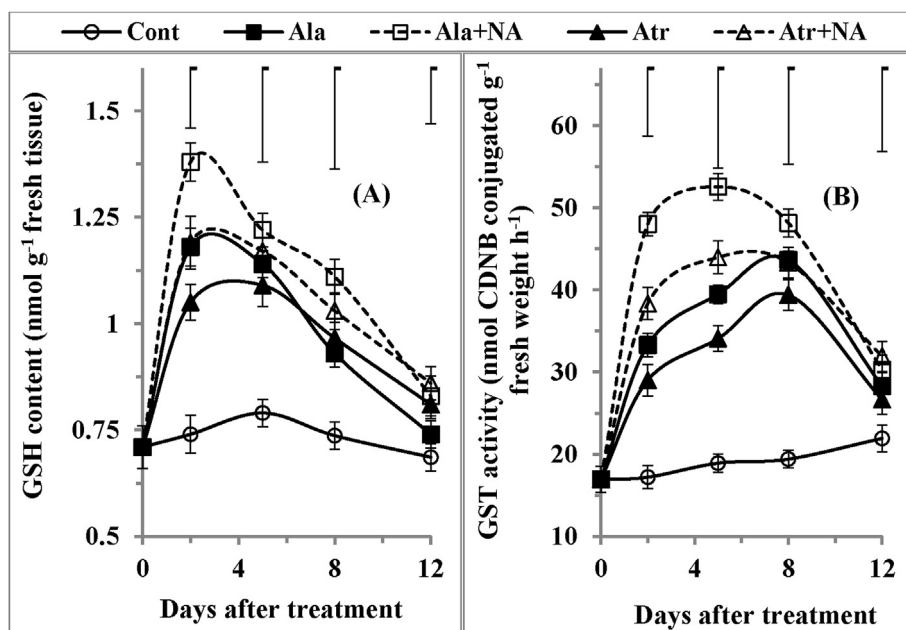
Figure 2. Influence of naphthalic anhydride (NA) on the content of glutathione (GSH) (A) and the activity of glutathione-S-transferase (GST) (B) in shoots of 10-day-old maize seedlings treated with alachlor (Ala) and atrazine (Atr) during the following 12 days. Values are means $\pm$ SD (n = 6). Vertical bars represent LSD at $p < 0.05$.
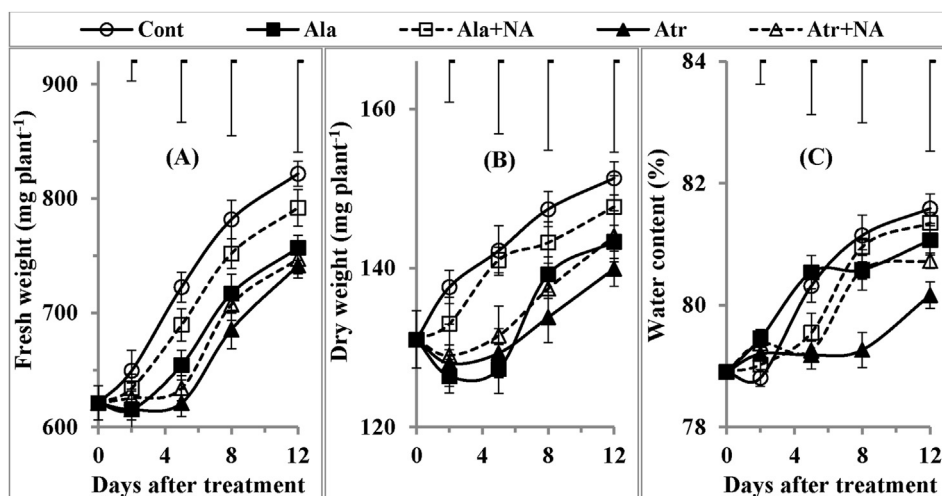

Figure 3. Influence of naphthalic anhydride (NA) on fresh weight (A), dry weight (B), and water content (C) of shoots of 10-day-old maize seedlings treated with alachlor (Ala) and atrazine (Atr) during the following 12 days. Values are means $\pm$ SD (n = 6). Vertical bars represent LSD at $p < 0.05$.

Ala and Atr significantly stimulated the activities of IAA-O and POD during the first 5 and 8 days, respectively, thereafter the effects became non-significant (Figure 5). However, the effect on POD was higher for Ala than Atr, the contrary was detected regarding IAA-O. Nevertheless, the application of NA levelled off the induced stimulation of the activities of IAA-O and POD enzymes. Upon using NA, the stimulations in IAA-O by herbicides became non-significant after the 2nd or the 5th day from treatment with Ala or Atr, respectively; however, both herbicides became with non-significant effects on POD after the 2nd day from treatment.

Meanwhile, the contents of phenolic compounds and anthocyanins were significantly elevated by treatment with Ala or Atr (Figure 6). The effects of both herbicides were most likely similar, both in magnitude and in duration. These effects were significant during the first 8 days then became non-significant thereafter. The dressing with NA led to great retractions in the increased levels of phenolic compounds and anthocyanins only after the 2nd day from treatment.

The activities of PAL, TAL and CI were significantly stimulated following treatment with Ala and Atr, the magnitude of stimulation in PAL and TAL was more pronounced in response to Ala than Atr, but the contrary was detected regarding CI (Figure 7). PAL activity in treated samples became comparable from the 5th or the 8th day from treatment with Ala or Atr, respectively, however both herbicides most likely became non-significant on TAL activity from the 5th day from treatment or on CI activity from the 8th day. The application of NA greatly retracted the herbicide-induced increases in the activities of PAL, TAL and CI. In spite of these retractions, NA overcame the effects of both herbicides not from the start but only after the 2nd onward and rendered them likely with no longer significant effect.

4. Discussion

NA is a safener protects plants from herbicide injuries by several means. It increases herbicide conjugation with GSH by GST (Deng and
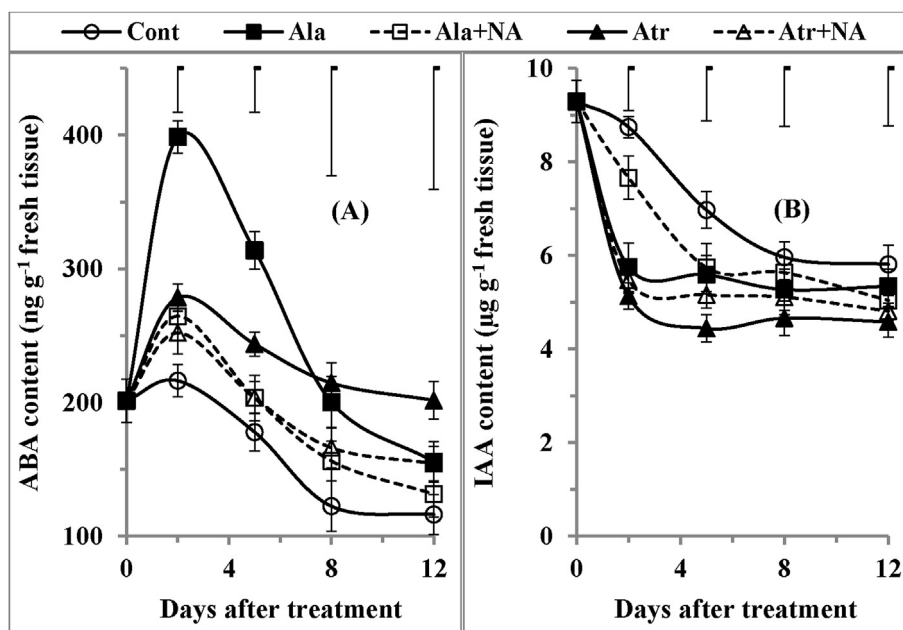
Figure 4. Influence of naphthalic anhydride (NA) on the content of ABA (A) and IAA (B) in shoots of 10-day-old maize seedlings treated with alachlor (Ala) and atrazine (Atr) during the following 12 days. Values are means ± SD (n = 6). Vertical bars represent LSD at $p < 0.05$.
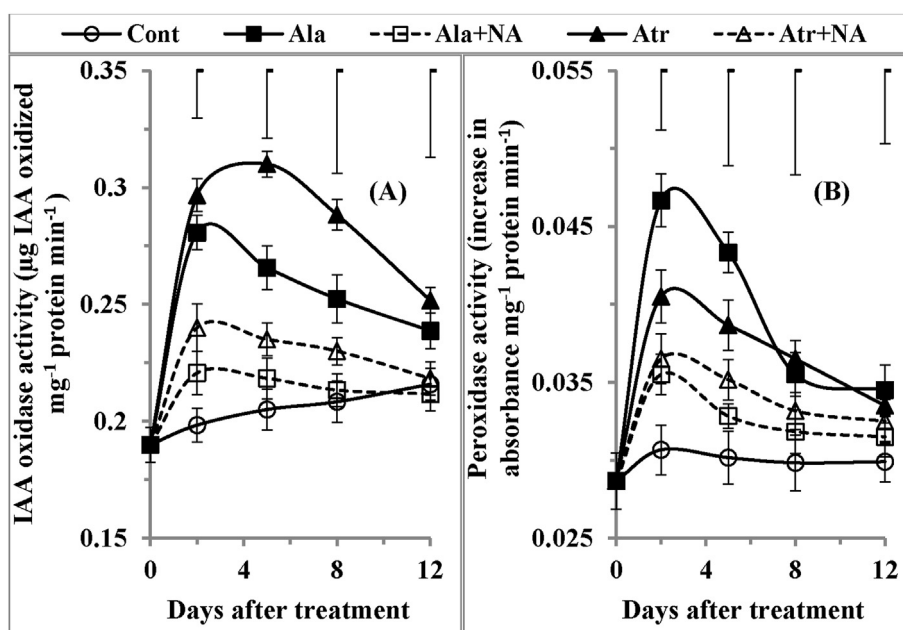

Figure 5. Influence of naphthalic anhydride (NA) on the activities of IAA-oxidase (A) and peroxidase (B) in shoots of 10-day-old maize seedlings treated with alachlor (Ala) and atrazine (Atr) during the following 12 days. Values are means ± SD (n = 6). Vertical bars represent LSD at $p < 0.05$.

Hatzios, 2003) or their hydroxylation by cytP450 (Dalazen et al., 2018). Moreover, Hwang et al. (1997) concluded that NA protects maize plants from the injury of bensulfuron-methyl or imazaquin at least partly through the enhancement of acetolactate synthase and acetyl CoA carboxylase activities, and the reduction of pyruvate accumulation. Recently, Krenchinski et al. (2019) found that NA protected photosystem II electron transport rate of common bean when using the herbicides bentazon, fluazifop-P + fomesafen, bentazon + imazamox, and cloransulam. However, other effects of NA could be included from which are the maintaining of plant growth regulators and secondary metabolites balances. In the present results, NA protected maize from the impacts of

Ala or Atr. Their presence in contact with the target sites of action in maize with greater persistence resulted in severe toxicity.

Herbicide persistence could result from high uptake and translocation than decomposition. As shown in the results, the residues of both herbicides were high and decreased with time elapse indicating the prevalence of the detoxification rate. The high residues detected on the 1st day were followed thereafter by retractions in magnitude concluding that the uptake and accumulation rates were high at first, then the detoxification started with the elapse of time leading to a consequent drop in these residues. Although there was detoxification, the rate seemed not efficiently enough to degrade both herbicides during the first few days of
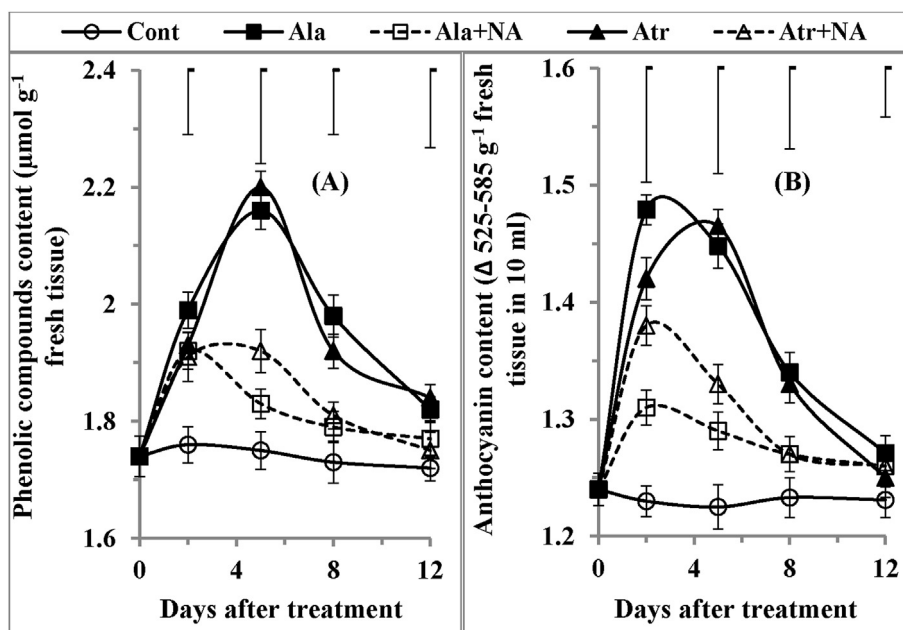
Figure 6. Influence of naphthalic anhydride (NA) on the content of phenolic compounds (A) and anthocyanins (B) in shoots of 10-day-old maize seedlings treated with alachlor (Ala) and atrazine (Atr) during the following 12 days. Values are means ± SD (n = 6). Vertical bars represent LSD at $p < 0.05$.
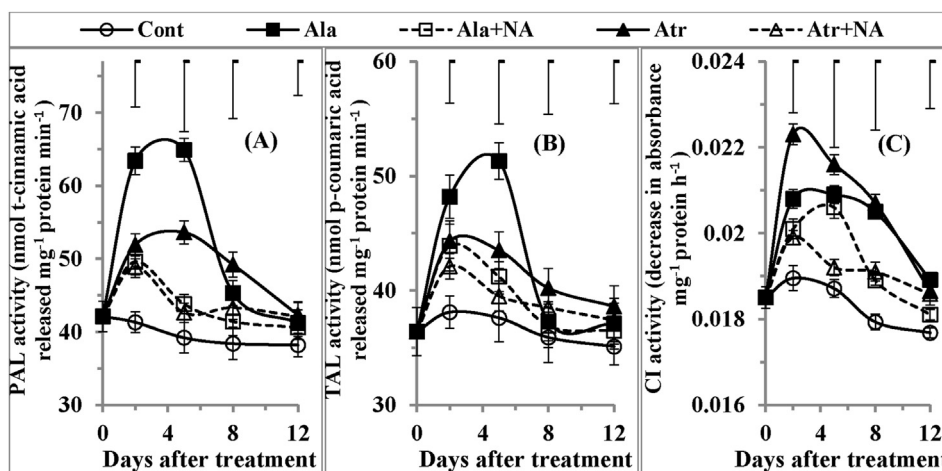

Figure 7. Influence of naphthalic anhydride (NA) on the activities of phenylalanine ammonia lyase (PAL) (A), tyrosine ammonia lyase (TAL) (B) and calchon isomerase (CI) (C) in shoots of 10-day-old maize seedlings treated with alachlor (Ala) and atrazine (Atr) during the following 12 days. Values are means ± SD (n = 6). Vertical bars represent LSD at $p < 0.05$.

germination. Therefore, exogenous supporters are needed as effective helper in mitigating the herbicide effects (Jang et al., 2019). The use of NA safener as seed-dressing could help seedlings to detoxify both herbicides. The safeners are considered an important tool for weed control in corn crop by increasing herbicides selectivity levels and safety (Maciel et al., 2012). Generally, the persistence of both herbicides, in the present results, showed some decreases with the elapse of time in the plant on its own, moreover NA supported the plant to induce further decreases in persistence. Such decreases would result less furiousness of the herbicide-induced stress status.

The main route of Ala and Atr detoxification is their conjugation with GSH by GST. Under herbicide treatment, maize was enforced to induce GSH and GST for enhancing the herbicide degradation rate. However, this degradation seemed insufficient to counterbalance the herbicide impacts. These findings were supported with the use of NA which provoked further inductions in GSH and GST particularly with Ala. Such induction would facilitate the opportunity towards herbicide conjugation

with GSH through the catalytic action of GST and so herbicide persistence was greatly retracted. Therefore, the increased GSH and GST could explain the enhancement of herbicide detoxification particularly with the application of NA. Some reports concluded that the GSTs are enhanced under certain conditions to increase the plant defense against several biotic and abiotic agents (Edwards et al., 2000; DeRidder et al., 2002; Nemat Alla and Hassan, 2006). Thus the activity of GST might contribute to the physiological selectivity of plants to tolerate herbicides. The present results revealed great increases in GST activities in maize by Ala and Atr coincided with increases in GSH levels suggesting fast and easy conjugation of herbicides to be tolerated by maize. In this regards, Stephensen et al. (2002) reported that GSH and GSH-related enzymes are important components in the defenses against stress in aerobic organisms. DeRidder et al. (2002) reported that herbicide safeners increased GSH content of *Arabidopsis* seedlings. They, moreover, concluded that safener-induced protection in cereals is associated with increased expression of herbicide detoxifying enzymes, including GST.

Consequently, the over production of GSH by NA, in the present results, would push to conjugate with Ala and Atr by GST and thereby to eliminate the herbicide phytotoxicity. So, the persistence of both herbicides decreased as GSH content and GST activity increased with a consequent enhanced tolerance of maize to the herbicides.

On the other hand, the magnitude of herbicide residues were synchronized with the reductions in growth parameters of maize shoots of treated samples. Growth reductions certainly appeared as a result of concomitant alterations in certain metabolic processes. The decrease in shoot fresh and dry weights reached a maximum of 9.4 and 10.5%, respectively by Ala and about 14 and 9.2%, respectively by Atr, nonetheless, the decrease in water content by either herbicide was slight. However, NA greatly eliminated the effects of Ala and Atr on growth parameters. In concomitant, the persistence of either herbicide was higher during the first 2 days but slightly retracted up to the 5th day for Ala or up to the 8th day for Atr then declined thereafter, the pattern was most likely in coincidence with the persistence of both herbicides. In this account, Maciel et al. (2012) concluded that the association of seed treatment with NA to isoxaflutole herbicide reduced significantly the herbicide impacts on corn, and provided higher yield. Moreover, Davies et al. (1998) indicated that NA significantly enhanced maize tolerance to the imidazolinone herbicide, AC 263222. They indicated that the protective effects of NA result from reduction of the herbicide translocation from root to shoot tissue and enhancement of its hydroxylation due to the stimulation of the activity of cytP450.

In addition, the growth of plants is mainly controlled by the balances in plant growth regulators, so the growth could be affected if the impacts of stress altered this balance. The results reported herein showed that IAA was significantly depressed by Ala and Atr while ABA was significantly increased relative to normal levels, the sequence of response followed that of residues supporting the changes in IAA and ABA are consequences of persisting herbicides. In this context, the levels of ABA in several plant species were found to be altered as a result of herbicide treatments. Zaric et al. (1983) found that Ala induced a very high increase in ABA- like substances in maize. Similarly, treatments with isoproturon also increased the levels of ABA in wheat (Nemat Alla and Hassan, 2014). Moreover, Hassan and Nemat Alla (2005) recorded increases in ABA levels of maize following treatment with rimsulfuron. ABA was shown to be implicated in plant acclimatization and protection against environmental stress involving the application of herbicides (Field and Caseley, 1987).

Therefore, the effect of Ala and Atr, observed in the present work on the growth of maize, might be a consequence of the herbicide-induced production of ABA that may play a role against the herbicide stress. On this account, Zeevaart and Creelman (1988) hypothesized that the accumulation of ABA might be a natural response that mediates the hardness of plants in response to environmental stress. Thus, the increased ABA levels in the present results could be regarded as an attempt for the plant to overcome the phytotoxic action of Ala and Atr herbicides. Meanwhile, Ala and Atr induced significant decreases in IAA only during the persistence of these herbicides, then the levels turned normal with the elapse of time as the herbicide residues decreased. Moreover, the activities of IAA-O and POD were stimulated in parallel with the drop in IAA suggesting their role in IAA degradation. In accordance, Nemat Alla and Hassan (2014) found that treatment of wheat with isoproturon induced reductions in IAA contents concomitant with stimulations in the activities of IAA-O and POD. The increased enzyme activities could explain the reduction in IAA levels.

Also, phenolic compounds and anthocyanins as well as the activities of PAL, TAL and CI were significantly stimulated by Ala than Atr. The pattern of increases in secondary metabolites and their enzymes was most likely similar to that of herbicide residues; elevated as the residues were high and likely decreased as the residues became low. These increases were induced by herbicides but retracted with the elapse of time. Secondary metabolites are considered as efficient antioxidants. They are a natural defense against biotic and abiotic events and so may increase

plant adaptation to stress conditions (Dorey et al., 1999). Ao et al. (2011) reported that the chemical constituents of *Smilax sebeana* contain six phenolic compounds potentially involved in antioxidant activity. Secondary metabolites are produced in plant tissues under the control of the secondary metabolism enzymes PAL, TAL and CI (Jez et al., 2000). These enzymes are considered as switching for secondary metabolism increased under certain conditions to increase the plant defense system (Gottstein et al., 1991). Some herbicides were found to induce increases in the activities of these enzymes; nevertheless, some other herbicides were inhibitory (Nemat Alla and Younis, 1995). The increased activities, in the present work by Ala and Atr, were accompanied by increases in phenolic compounds and anthocyanins. On the other hand, NA mostly decreased the content of secondary metabolites and enzyme activities indicating the withdrawn of the stress status. These changes in phenolics, anthocyanins and activities of the secondary metabolism enzymes were likely similar giving an explanation for the withstanding of maize to cope with herbicide toxicity by the application of NA. Consequently, NA supported maize to insist the persistence of Atr and Atr to great extent and thereby overcame their toxicity with an elevation in the tolerance of the plant.

In conclusion, Ala and Atr residues were detected in maize shoots following treatment with both herbicides; the persistence of Atr was greater than Ala. Meanwhile, significant inductions were detected in GSH and GST; the effect of Ala was higher than Atr. On the contrary, growth parameters were significantly reduced. Also, IAA was decreased in coincidence with enhancement in IAA-O and POD activities while ABA was increased concurrently with induction in phenolics and anthocyanins and stimulation of PAL, TAL and CI activities. The immediate detection of herbicide residues could conclude that their effects are consequences of their persistence. However, the herbicide-induced increases in ABA and decreases in IAA in concomitant with elevating secondary metabolites and their enzymes confirm the occurrence of an imposed stress status in treated seedlings. On the other hand, the stimulation in IAA-O and POD activities preceded the drop of IAA whereas the increases in secondary metabolites followed the stimulation in PAL, TAL and CI activities declaring the regulatory roles of these enzymes in IAA and secondary metabolites. The application of NA ameliorated growth parameters coincidently with decreasing in the herbicide residues in concomitant with increases in GSH and GST and maintaining phytohormones and secondary metabolism. These findings could suggest that NA elevated tolerance of maize to Ala and Atr through lowering the herbicide persistence and fixing balances of phytohormones and secondary metabolites.

Declarations

Author contribution statement

Mamdouh M. Nemat Alla: Conceived and designed the experiments; Analyzed and interpreted the data; Wrote the paper.

Nemat M. Hassan: Conceived and designed the experiments; Performed the experiments; Contributed reagents, materials, analysis tools or data; Wrote the paper.

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