Comprehensive evaluation of fluroxypyr herbicide on physiological parameters of spring hybrid millet

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Foxtail millet (*Setaria italic* L.) is an important food and fodder crop that is cultivated worldwide. Quantifying the effects of herbicides on foxtail millet is critical for safe herbicide application. In this study, we analyzed the effects of different fluroxypyr dosages on the growth parameters and physiological parametric of foxtail millet, that is, peroxidation characteristics, photosynthetic characteristics, and endogenous hormone production, by using multivariate statistical analysis. Indicators were screened via Fisher discriminant analysis, and the growth parameters, peroxidation characteristics, photosynthesis characteristics and endogenous hormones of foxtail millet at different fluroxypyr dosages were comprehensive evaluated by principal component analysis. On the basis of the results of principal component analysis, the cumulative contribution rate of the first two principal component factors was 93.72%. The first principal component, which explained 59.23% of total variance, was selected to represent the photosynthetic characteristics and endogenous hormones of foxtail millet. The second principal component, which explained 34.49% of total variance, represented the growth parameters of foxtail millet. According to the principal component analysis, the indexes were simplified into comprehensive index Z, and the mathematical model of comprehensive index Z was set as $F = 0.592Z_1 + 0.345Z_2$. The results showed that the comprehensive evaluation score of fluroxypyr at moderate concentrations was higher than at high concentrations.

Consequently, 1 L (active ingredient, ai) ha^{-1} fluroxypyr exerted minimal effects on growth parameters, oxidase activity, photosynthetic activity, and endogenous hormones, and had highest value of comprehensive evaluation, which had efficient and safe benefits in foxtail millet field.

1   **Comprehensive evaluation of fluroxypyr herbicide on physiological**

2   **parameters of spring hybrid millet**

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7   # ABSTRACT

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9   Quantifying the effects of herbicides on foxtail millet is critical for safe herbicide application. In this study, we

10   analyzed the effects of different fluroxypyr dosages on the growth parameters and physiological parametric of

11   foxtail millet, that is, peroxidation characteristics, photosynthetic characteristics, and endogenous hormone

12   production, by using multivariate statistical analysis. Indicators were screened via Fisher discriminant analysis,

13   and the growth parameters, peroxidation characteristics, photosynthesis characteristics and endogenous

14   hormones of foxtail millet at different fluroxypyr dosages were comprehensive evaluated by principal

15   component analysis. On the basis of the results of principal component analysis, the cumulative contribution

16   rate of the first two principal component factors was 93.72%. The first principal component, which explained

17   59.23% of total variance, was selected to represent the photosynthetic characteristics and endogenous

18   hormones of foxtail millet. The second principal component, which explained 34.49% of total variance,

19   represented the growth parameters of foxtail millet. According to the principal component analysis, the indexes

20   were simplified into comprehensive index Z, and the mathematical model of comprehensive index Z was set as

21    $F = 0.592Z_1 + 0.345Z_2$. The results showed that the comprehensive evaluation score of fluroxypyr at moderate

22 concentrations was higher than at high concentrations. Consequently, 1 L (active ingredient, ai) ha^{-1}

23 fluroxypyr exerted minimal effects on growth parameters, oxidase activity, photosynthetic activity, and

24 endogenous hormones, and had highest value of comprehensive evaluation, which had efficient and safe

25 benefits in foxtail millet field.

INTRODUCTION

27 Foxtail millet (*Setaria italica* L.) is a valuable economic crop because it is rich in protein and crude fat

28 and used as a staple food worldwide. This plant has seven essential amino acids, and its contents are higher

29 than those found in other crops. In particular, methionine and tryptophan, which are important in preventing

30 atherosclerosis and softening the blood vessel, are abundant in this plant. However, weed infestation has

31 severely limited foxtail millet production in China (Guo et al., 2018). Weeds are the most important biotic

32 factor affecting agricultural production; they are responsible for over 55.56% of total foxtail millet yield losses

33 (Zhou et al., 2012). For weed control, herbicide application is considered the most cost-efficient and effective

34 method (Rubin, 1996). Rational application of herbicides, such as monosulfuron, monosulfuron mix propazine,

35 2,4-D, and prometryn, can effectively control weeds in foxtail millet fields; however, these herbicides readily

36 cause phytotoxicity reaction (Tian & Wang, 2010). Thus, finding suitable herbicides for use in foxtail millet

37 field is a considerable challenge in weed control.

38 Fluroxypyr (4-amino-3, 5-dichloro-6-fluoro-2-pyridyloxyacetic acid) is originally applied in cereal, olive tree, and

39 fallow cropland fields, to control annual or perennial weeds (Hellou et al., 2009). These herbicides cause auxin

40 overdose or excessive endogenous auxin concentrations, thereby resulting in an imbalance of auxin

41 homeostasis and interaction with other hormones in tissues, which ultimately cause the succeeding series of

42 biochemical and physiological processes associated with herbicide action (Grossmann, 2010). Liu (2014)

43   demonstrated that the label fluroxypyr dose can be used in maize (*Zea mays*) and winter wheat (*Triticum*

44   *aestivum*) fields, and has desirable control effects on broadleaf weeds. Considering the significant differences

45   in the sensitivity of crop varieties to fluroxypyr, it is necessary to indicate whether fluroxypyr is safe for use on

46   foxtail millet.

47   Foxtail millet tolerance toward herbicides depends on either the alteration of the target site (TS) or non-

48   target site (NTS) mechanisms. TS resistance mechanism is manifested by structural changes due to point

49   mutations in herbicide-binding proteins, such as the D1 protein in the photosystem II (PSII) complex (Thiel &

50   Varrelmann, 2014), acetolactate synthase (Tranel, Wright & Heap, 2014), acetyl-CoA carboxylase (Kaundun,

51   2014), or 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS) (Sammons & Gaines, 2014). TS resistance

52   mechanism is occasionally the result of an increased number of copies of the target gene, such as EPSPS

53   (Gaines et al., 2010; Vila-Aiub et al., 2014). The NTS mechanism involves herbicide detoxification by

54   glutathione S-transferase or cytochrome P450 monooxygenase, reduced absorption or translocation in the plant

55   and sequestration into vacuoles (Matzrafi et al., 2014). In contrast to TS, NTS resistance mechanisms can

56   occasionally be more widespread and exhibit resistance to many herbicides. However, information regarding

57   the physiological mechanism of Zhangzagu hybrid millet after the application of fluroxypyr is limited. Thus,

58   the NTS resistance mechanisms to fluroxypyr in spring hybrid millet should be understood.

59   The hybrid foxtail millet 'Zhangzagu' is popular, especially Zhangza 5, with a large production area in

60   China due to its high yield, drought resistance, and nutritional value (Dong et al., 2014). Thus, we used

61   Zhangza 5 as the model species for the present study, especially for the study of the physiological mechanism

62   due to tolerance against fluroxypyr. Herein, the objective of this study are as follows: (1) to comprehensively

63   investigate the influence of different dosages of fluroxypyr on the growth and physiological mechanism of

64   spring hybrid millet; (2) to further understand the NTS resistance mechanisms to fluroxypyr-induced oxidative

65   stresses; (3) to compare and analyze the main factors contributing to the effect of fluroxypyr on spring hybrid

66   millet growth. Thus, we provided convincing evidence for the correlations of NTS resistance to fluroxypyr

67   exposure, and aimed at obtaining the optimal fluroxypyr application dose for spring hybrid millet.

68   ## MATERIALS AND METHODS

69   **Plant and experimental design**

70   Fluroxypyr (20%, emulsifiable concentrate) was provided by Dow AgroScience Co. (Jiangsu, China).

71   Foxtail millet (*Setaria italica* L. cv. Zhangza 5) was supplied by the Zhangjiakou Academy of Agricultural

72   Sciences of Hebei Province, China. The seeds were uniformly sown in a plastic pot (130 mm diameter)

73   containing a mixture of sand and soil (1:2, v:v). Calcareous cinnamon soil, with a pH of 7.85, 24.49 g kg^{-1}

74   organic matter, 51.92 mg kg^{-1} total nitrogen, 24.13 mg kg^{-1} available phosphorus, and 183.6 mg kg^{-1} rapidly-

75   available potassium was used in this work. At the three-leaf stage, the foxtail millet seedlings were thinned and

76   maintained at 10 uniform plants per pot.

77   The experiment was conducted as a randomized complete block design with three replications. After

78   growing to the five-leaf stage, foxtail millet seedlings were treated with 0, 0.5, 1, 2, and 4 L ai ha^{-1} fluroxypyr

79   by using a laboratory pot-sprayer equipped with a nozzle and previously calibrated to deliver 450 L ha^{-1}. The

80   manufacturer recommended an effective dose of 1 L ai ha^{-1} for field application. After herbicide treatment for

81   5 and 10 days, the physiological indices of all foxtail millet seedlings were analyzed.

82   **Measurement of growth parameters**

83   The plant height, length and width of leaves were measured with a ruler. Leaf area was calculated using the

84   following equation: leaf area = 0.75 × leaf length × leaf width. Leaf width was considered the widest part of

85   the penultimate leaf.

86   **Determination of superoxide generation rate and H$_2$O$_2$ content**

87   Fresh foxtail millet leaves (0.1 g) were homogenized with 2 mL of 65 mmol L^{-1} sodium phosphate buffer

88   (pH 7.8) and centrifuged at 10,000 rpm for 10 min. The supernatant (1 mL) was mixed with 1 mL of 65 mmol

89   L^{-1} sodium phosphate buffer (pH 7.8) and 0.2 mL of 10 mmol L^{-1} hydroxyl ammonium chloride. The resulting

90   supernatant was incubated 25°C for 20 min, and the above reaction mixture (1 mL) was mixed with 1 ml of 4-

91   aminobenzene sulfonic acid (17 mmol L^{-1}) and 1 mL of α-naphthylamine (7 mmol L^{-1}), then incubated at 30

92   °C for 30 min. The absorbance at 530 nm was measured with a *756C-UV-VIS* spectrophotometer (Shanghai

93   Spectrum Instruments Co. Ltd) (Elstner & Heupel, 1976).

94   Fresh foxtail millet leaves (0.1g) were homogenized with 5 mL of chilled acetone in an ice bath and

95   centrifuged at 4,000 rpm for 15 min. The supernatant (1 mL) was mixed with 0.1 mL of 20% TiCl$_4$

96   concentrated hydrochloric acid and 0.2 mL of concentrated ammonium hydroxide. The reaction mixture was

97   centrifuged at 10,000 rpm for 10 min and the precipitate was dissolved in 3 mL H$_2$SO$_4$ (1 mol L^{-1}). The

98   absorbance of the supernatant at 410 nm was monitored with a *756C-UV-VIS* spectrophotometer (Zhang et al.,

99   2012).

100   **Determination of antioxidant enzyme activities**

101   Fresh foxtail millet leaves (0.1g) were homogenized with 2 mL of sodium phosphate buffer (pH 7.0,

102   containing 0.1 mmol L^{-1} EDTA and 1% PVP (w/v)) in an ice bath. The homogenate was centrifuged at 10,000

103   rpm for 15 min at 4°C. The supernatant was extracted and used to measure the activities of superoxide (SOD),

104   peroxidase (POD), catalase (CAT), and ascorbate peroxidase (APX). The samples were measured with a *756C-*

105   *UV-VIS* spectrophotometer.

106   SOD activity was analyzed by using the nitro blue tetrazolium (NBT) method. The reaction mixture (5 mL)

107   consisted of phosphate buffer (50 mmol L^{-1}, pH 7.8), L-methionine (13 mmol L^{-1}), NBT (0.075 mmol L^{-1}),

108   EDTA (0.1 mmol L^{-1}), riboflavin (0.002 mmol L^{-1}), and 20 μL of enzyme extract. The reaction mixture was

109   then illuminated under 4000 lx for 15 min at 25°C. One unit of activity corresponds to the amount of protein

110   required to inhibit 50% of the initial reduction of NBT under light conditions. The absorbance at 560 nm was

111   measured, and a non-irradiated complete reaction mixture served as the control. For POD activity

112   determination, 20 μL of enzyme extract was added into 3 mL of reaction liquid containing 3 mL of sodium

113   phosphate buffer (100 mmol L^{-1}, pH 6.0), 19 μL of guaiacol, and 28 μL of 30% H_2O_2. POD activity was

114   measured by the changes in the absorbance of the reaction solution at 470 nm for 3 min. For CAT activity

115   determination, the reaction liquid consisted of 2.7 mL of Tris-HCl (50 mmol L^{-1}, pH 7.0), 50 μL of H_2O_2 (200

116   mmol L^{-1}), and 20 μL of enzyme extract. Enzyme activity was continuously determined at 240 nm for 3 min

117   following H_2O_2 decomposition. In accordance with the method of APX activity determination by Nakano &

118   Asada (1981), the reaction mixture consisted of 3 mL sodium phosphate buffer (50 mmol L^{-1}, pH 7.0), 0.4 mL

119   of EDTA (0.3 mmol L^{-1}), 1 mL of ascorbate (0.9 mmol L^{-1}), and 1 mL of enzyme extract. The reaction

120   mixture was then incubated for 5 min at 25 °C. The above reaction mixture was mixed with 0.5 mL of H_2O_2

121   (0.25 mmol L^{-1}), and H_2O_2 decomposition was measured by the decline in absorbance at 290 nm for 1 min.

122   Glutathione reductase (GR) activity was analyzed according to Halliwell & Foyer (1978). Fresh foxtail

123   millet leaves (0.1g) were ground in an ice bath with 2 mL of Tris-HCl (pH 7.5, containing 1% PVP and 0.1

124   mmol L^{-1} EDTA), and centrifuged at 10,000 rpm for 15 min at 4°C. The enzyme extract (150 μL) was added

125   into 3 mL of reaction liquid including Tris-HCl (pH 7.5), $MgCl_2$ (3 mmol L^{-1}), GSSG (0.5 mmol L^{-1}), and

126   NADPH (0.15 mmol L^{-1}). Enzyme activity was calculated by the change in absorbance at 340 nm for 3 min.

127 **Determination of photosynthetic gas exchange and chlorophyll**

128 Photosynthetic rate (P_N), transpiration rate (E), and stomatal conductance (G_s) were measured by CI-340

129 portable photosynthesis system (CID Bio-Science, Inc., USA) from 9:30 to 10:30 am. The photosynthetically

130 active radiation (PAR) at the leaf surface was approximately 11000 ± 50 μmol/m²/s, the temperature of the leaf

131 chamber was 30 ± 2 °C, and the ambient CO_2 concentration was 380 ± 50 μmol/mol.

132 To measure photosynthetic pigments, fresh leaves (0.1g) were soaked in 10 mL of ethanol (96%, v/v) and

133 stored in the dark for 24 h. The absorbance of the supernatants was measured at 649 and 665 nm with a *756C-*

134 *UV-VIS* spectrophotometer (Lichtenthaler, 1987).

135 **Measurement of chlorophyll fluorescence and P$_{700}$ parameters**

136 The chlorophyll fluorescence and P$_{700}$ parameters were simultaneously detected using a luminoscope (Dual

137 PAM-100, WALZ, Germany) (Pfündel, Klughammer & Schreiber, 2008). After dark adaptation for 30 minutes,

138 the kinetics of chlorophyll fluorescence induction and P700 oxidation were simultaneously detected according

139 to the "Fluo + P$_{700}$" analysis mode.

140 First, the minimal fluorescence (F_0) was detected under weak light (7 μmol/m²/s). Subsequently, the

141 maximum fluorescence (F_m) was determined by the saturation pulse (4000 μmol/m² /s, 800 ms) method. The

142 slow induction curve was calculated as follows: $F_v/F_m = (F_m - F_0)/F_m$, $ETR_{II} = PAR \times 0.84 \times 0.5 \times Y_{II}$, $Y_{II} =$

143 $(F_m' - F)/F_m'$, $Y_{NO} = 1/(NPQ + 1 + qL (F_m/F_0 - 1))$, $Y_{NPQ} = 1 - Y_{II} - Y_{NO}$, where F_v/F_m and Y_{II} are the

144 maximum PSII quantum yield and effective PSII quantum yield, respectively, and Y_{NPQ} and Y_{NO} are the

145 regulated energy dissipation and non-regulated energy dissipation, respectively.

146 P$_{700}$ oxidation was monitored by the changes in the transmittance signals at 875 nm and 830 nm

147 (Klughammer & Schreiber, 2008). The maximal P$_{700}$ (P$_m$) was denoted by the maximal P$_{700}$ signal reduction to

148   full oxidation. Y_{NA}, the nonphotochemical quantum yield of PSI, is a measure of the acceptor-side limitation

149   and calculated according to the formula $Y_{NA} = (P_m' - P_m)/P_m'$. Y_I, the photochemical quantum yield of PSI,

150   was calculated according to the formula $Y_I = (P_m' - P)/P_m$. Y_{ND}, the nonphotochemical quantum yield of PSI, is

151   a measure of the donor side limitation and calculated as $Y_{ND} = (P - P_0)/P_m$. The sum of these three quantum

152   yields is one, i.e.: $Y_I + Y_{ND} + Y_{NA} = 1$. The electron transfer efficiency of PSI, ETR_I was assessed by the Dual

153   PAM software.

Measurement of endogenous hormones

155   Fresh foxtail millet leaves (0.5 g) were homogenized with 4 mL of 80% methanol (1 mmol/L 2, 6-di-tert-

156   butyl-4-methylphenol) in an ice bath. The homogenate mixture was refrigerated at 4 °C for 4 h, and

157   centrifuged at 3,500 rpm for 8 min. The precipitate was added to 1 mL of 80% methanol, refrigerated at 4 °C

158   for 1 h, and centrifuged. The supernatant passed through a C_{18} solid phase extraction column, and then dried

159   with nitrogen. The hormone extract was obtained by mixing with 1 mL of the sample diluent (PBS containing

160   0.1% Tween-20, gelatin 1 μg/L) and determined using enzyme-linked immunosorbent assay. The hormone

161   assay kit was supplied by China Agricultural University, and the content of auxin (IAA), abscisic acid (ABA),

162   zeatin (ZR), and gibberellin (GA) contents were measured by using the Thermo Multiskan FC enzyme-labeled

163   instrument.

Statistical analysis

165   Statistical analyses were performed using Statistical Product and Service Solutions 19.0 (SPSS Inc.,

166   Chicago, IL). Quantitative data were expressed as the mean± standard error, and multiple comparisons were

167   analyzed by Duncan's multiple range test. $P < 0.05$ was considered significantly different. In this study, Fisher

168   discriminant and principal component analyses (PCA) were calibrated by using the selected indices with the

169 highest difference between the different herbicide treatments and control, to simplify the indicators.

RESULTS

Effects of fluroxypyr on growth parameters

172 After exposing the seedlings to 0.5–4.0 L ai ha^{-1} fluroxypyr for 5 days, the plant height of Zhangza 5

173 significantly differed between the control and herbicide treatments. At 10 days post-fluroxypyr treatment, the

174 plant height increased by 10.16%, 12.62%, and 5.73% after exposure to 0.5, 1.0, and 2.0 L ai ha^{-1}, respectively.

175 As shown in Table 1, the leaf area of Zhangza 5 significantly differed between the recommended dose (1 L ai

176 ha^{-1}) and other treatments after 5 days. A similar response of the leaf area was recorded when Zhangza 5 was

177 exposed to fluroxypyr for 10 days, thereby showing significant difference among treatments compared with the

178 recommended dosage.

Effects of fluroxypyr on stress parameters

180 Fluroxypyr treatment at doses ranging from 0 to 4 L ai ha^{-1} increased H_2O_2 and O_2^- accumulation in

181 plants, with the maximum expression levels obtained after treatment with a dosage of 4 L ai ha^{-1} for 5 and 10

182 days. As shown in Table 2, maximum H_2O_2 and O_2^- accumulations significantly increased by treatment with 4

183 L ai ha^{-1} fluroxypyr for 5 and 10 days compared with the control. Similarly, treatment with fluroxypyr at the

184 recommended dose (1 L ai ha^{-1}) increased H_2O_2 and O_2^- contents by 1.01- and 1.02- fold relative to the control

185 after 5 days and by 2.02- and 1.05-fold relative to the control after 10 days, respectively. In our studies, we

186 observed a time-dependent decrease in reactive oxygen species (ROS) in response to fluroxypyr exposure.

Effects of fluroxypyr on enzyme activities

188 As shown in Figure 1, the SOD, POD, CAT, APX, and GR activities significantly increased. The

189 exposure of Zhangza 5 to increasing levels of fluroxypyr led to increases in SOD, CAT, APX, and GR

190   activities. However, further increases in fluroxypyr concentration beyond 2 L ai ha^{-1} failed to promote SOD,

191   CAT, APX, and GR accumulation. The activities of SOD, CAT, APX, and GR significantly increased after

192   treatment with 2 L ai ha^{-1} fluroxypyr relative to the control. POD activities were stimulated after exposure to

193   different fluroxypyr concentrations, and its effect was concentration-dependent; the highest POD activity was

194   observed at 4 L ai ha^{-1} fluroxypyr. POD activities significantly increased by 2.91- and 3.53- fold relative to the

195   control after treatment with 4 L ai ha^{-1} fluroxypyr for 5 and 10 days, respectively.

196   **Effects of fluroxypyr on net photosynthetic rate and pigments**

197   As shown in Table 3, P_N, E, G_s, and *Chl* showed a progressive decrease with increasing fluroxypyr

198   concentration. After 1 L ai ha^{-1} (recommended dose) fluroxypyr treatment for 5 days, P_N and E significantly

199   decreased by 23.72% and 22.56% compared with the control, respectively. However, 1 L ai ha^{-1} fluroxypyr

200   treatment for 5 days inhibited G_s by 3.53%. Moreover, after with 1 L ai ha^{-1} fluroxypyr for 10 days, P_N, E, and

201   G_s of the treated plants were reduced by 18.78%, 15.12%, and 18.79% compared with the control, respectively.

202   According to our results, significant differences were recorded between the treatments and control.

203   Chlorophyll content was also significantly affected following fluroxypyr treatment; however, no significant

204   differences were observed between the control and treatment using the recommended dose after 5 and 10 days

205   of exposure.

206   **Effects of fluroxypyr on chlorophyll fluorescence and P$_{700}$ parameters**

207   After 5 days of exposure to fluroxypyr, the F_v/F_m of treated plants decreased to 0.13%, 0.32%, 1.71%, and

208   3.29% compared with the control, and the differences between treatments were insignificant. Moreover, after

209   10 days, the F_v/F_m of the treated plants recovered to control levels. As shown in Figure 2, the changes in Y(II)

210   and ETR(II) were consistent under fluroxypyr treatment, and the values were reduced in a dose-dependent

211   manner. After 5 and 10 days of treatment, Y(II) decreased from 12.44% to 22.96%, and from 18.25% to

212   32.14%, respectively, and ETR(II) decreased from 12.37% to 22.69%, and from 14.91% to 32.31%,

213   respectively. Additionally, Y(NPQ) increased with increasing fluroxypyr doses, and the maximum

214   accumulation was observed at 2 L ai ha^{-1}. During treatment with 2 L ai ha^{-1} fluroxypyr, the Y(NPQ) values

215   were 36.39%, and 50.75%, compared with the control.

216   PSI activities were substantially affected by fluroxypyr treatment. As shown in Figure 2, Pm, Y(I), and

217   ETR(I) were evidently decreased with increasing fluroxypyr dosage; however, no significant differences

218   between the control and herbicide-treated plants were observed after 5 days of exposure. Treatment with

219   increasing fluroxypyr concentration increased the Y(ND) of Zhangza 5. The maximum Y(ND) level was

220   observed at fluroxypyr treatment. However, further increasing the dosages of fluroxypyr beyond 2 L ai ha^{-1} no

221   longer increased Y(ND).

222   **Effects of fluroxypyr on endogenous hormones**

223   Fluroxypyr treatment at doses ranging from 0.5 to 4 L ai ha^{-1} increased IAA and ABA accumulation in

224   plants, with the maximum accumulation observed at 4 L ai ha^{-1} (Figure 3). The application of 4 L ai ha^{-1}

225   fluroxypyr significantly increased IAA and ABA by 26.69% and 23.36% relative to that of the control after 5

226   days of treatment and 5.37% and 35.53% relative to that of the control after 10 days of treatment, respectively.

227   A similar response was observed between GA and ZR accumulation in Zhangza 5 exposure to fluroxypyr; GA

228   and ZR decreased with increasing fluroxypyr concentration, and the difference observed between the control

229   and treated plants was significant. After treatment with 1 L ai ha^{-1} (recommended dose) fluroxypyr for 5 and

230   10 days, the GA and ZR values significantly decreased by 10.46%, 18.72%, and by 13.50%, 23.59% relatively

231   to the control treatment, respectively.

232   **Comprehensive evaluation of fluroxypyr on various foxtail millet indices**

233   To further comprehensively evaluate the effects of fluroxypyr on various foxtail millet indices, Fisher

234   discriminant analysis was used. Herbicide treatment was used as the grouping variable Y, and the

235   physiological parameters were used as the independent variable X.

236   (1) Fisher discrimination was used to evaluate the seven indicators of foxtail millet leaf, namely,

237    $H_2O_2(X_1)$, O_2^- (X_2), $SOD(X_3)$, $POD(X_4)$, $CAT(X_5)$, $APX(X_6)$, and $GR(X_7)$. The following discriminant

238   functions were obtained:

239    $Y_1 = -3.850 + 4.868X_3 + 3.588X_6 - 48.734X_7$

240    $Y_2 = -0.344 - 1.408X_3 + 2.886X_6 - 7.736X_7$

241    $Y_3 = -5.062 - 0.107X_3 + 0.020X_6 + 12.487X_7$

242   The cumulative contribution rate of the first-class functions, which is the main function, reached 98.8%.

243   Thus, $SOD(X_3)$, $POD(X_4)$, and $GR(X_7)$ were used as indices to evaluate the peroxidation characteristics of

244   foxtail millet.

245   (2) Fisher discrimination was used to evaluate the fourteen indicators of foxtail millet leaf, namely,

246    $P_N(X_1)$, $E(X_2)$, $G_s(X_3)$, $Chl(X_4)$, $F_v/F_m(X_5)$, $Y(II)(X_6)$, $ETR(I)(X_7)$, $Y(NPQ)(X_8)$, $Y(NO)(X_9)$, $P_m(X_{10})$,

247    $Y(I)(X_{11})$, $ETR(I)(X_{12})$, $Y(ND)(X_{13})$, and $Y(NA)(X_{14})$. The following discriminant functions were obtained:

248    $Y_1 = -252.994 + 427.640X_6 - 66.364X_8 + 143.154X_{10} + 1.396X_{12}$

249    $Y_2 = -69.158 + 151.904X_6 + 96.548X_8 + 22.743X_{10} - 0.036X_{12}$

250    $Y_3 = -5.251 - 91.400X_6 - 1.385X_8 + 7.042X_{10} + 0.184X_{12}$

251    $Y_4 = 1.257 + 2.323X_6 - 1.378X_8 + 3.767X_{10} - 0.018X_{12}$

252   The cumulative contribution rate of the first-class functions, which is the main function, reached 94.4%.

253   Thus, Y(II)(X$_6$), Y(NPQ)(X$_8$), P$_m$(X$_{10}$), and ETR(I)(X$_{12}$) were used as indices to evaluate the photosynthetic

254   characteristics of foxtail millet.

255   (3) Fisher discrimination was used to evaluate the four indicators of foxtail millet leaf, namely, IAA(X$_1$),

256   GA(X$_2$), ZR(X$_3$), and ABA(X$_4$). The following discriminant functions were obtained:

257        $Y_1 = -78.315 + 8.217X_2 + 0.229X_4$

258        $Y_2 = -13.841 + 1.139X_2 + 0.073X_4$

259   The cumulative contribution rate of the first-class functions, which is the main function, reached 94.4%.

260   Thus, GA(X$_2$) and ABA(X$_4$) were used as indices to evaluate the endogenous hormone characteristics of

261   foxtail millet.

262   As shown in Table 4, the cumulative contribution rate of the first two principal components reached

263   93.72%. Thus, the first two principal components were further analyzed. The first principal component

264   accounted for 59.23% of the variance observed, whereas the second principal component accounted for

265   34.49% of this variance. The load values of ERT (I), Y (II), and GA, which were 0.978, 0.975 and 0.963,

266   respectively, were the largest in the first principal component. ABA also showed high absolute loading value

267   in the first principal component, but exhibited a negative effect. Therefore, the first principal component could

268   explain the photosynthetic characteristics and endogenous hormones of foxtail millet. In the second principal

269   component, the loading values of plant height and leaf area were relatively high and could represent the growth

270   parameters of foxtail millet.

271   According to the principal component analysis, the model was set up as follows: F = 0.592Z$_1$ + 0.345Z$_2$.

272   The comprehensive evaluation scores of the 2 and 4 L ai ha^{-1} fluroxypyr treatments were relatively low, which

273   indicated that high fluroxypyr concentrations negatively affected the photosynthetic characteristics and

274   endogenous hormones of foxtail millet. Furthermore, the comprehensive evaluation scores of the 0.5 and 1 L ai

275   ha^{-1} fluroxypyr treatments were 0.941 and 2.007, respectively, and the scores of first two principal components

276   were positive, which indicated that the moderate concentrations of fluroxypyr positively affected the

277   peroxidation characteristics, photosynthetic characteristics, and endogenous hormones of foxtail millet (Table

278   5). Consequently, the scores of the two factors in 1 L ai ha^{-1} fluroxypyr treatments were both relatively high

279   and represented the comprehensive effect of herbicide in foxtail millet (Figure 4).

280   **DISCUSSION**

281   Numerous studies have demonstrated that excessive application of fluroxypyr in the environment inhibits

282   plant growth (Guo et al., 2010; Guo et al., 2018). In the present study, we found that fluroxypyr had a slight

283   effect on plant growth at relatively low doses, but exerted significant influence at high doses. The growth

284   inhibition of fluroxypyr is closely related to the accumulation of O_2^- and H_2O_2 in plants (Guo et al., 2010).

285   According to the inhibitory effect of fluroxypyr on ROS, the present study showed that the maximum

286   accumulation of H_2O_2 and O_2^- occurs when plants are treated with 4 L ai ha^{-1} fluroxypyr for 5 and 10 days.

287   Herbicide-induced oxidative stress causes cellular peroxidation and molecular damage through ROS over-

288   accumulation (Kehrer, 1993; Chen et al., 2009). To inhibit ROS action, plants have evolved protective

289   enzymatic mechanisms, such as those involving SOD, POD and CAT (Khan & Kour, 2007). In most cases, the

290   enzyme-based antioxidant system is one of the important ways for plants to resist environmental stress, which

291   reflects not only the level of toxicity but also the ability to tolerate stress. Our analyses showed that the SOD,

292   POD, CAT, APX, and GR activities generally increased at low fluroxypyr dosages but decreased at high

293   fluroxypyr dosages, thereby reflecting an increased degree of oxidative stress.

294   Several symptoms related to changes in plant photosynthesis were observed in this work. After fluroxypyr

295   treatment, one such visible symptom was chlorosis, which showed that chlorophyll was certainly sensitive to

296   fluroxypyr exposure. In the present study, chlorophyll formation was considerably suppressed at high

297   concentrations (2 and 4 L ai ha^{-1}). A reduction in P_N, as reflected by the decrease in E and G_s, was also noted

298   after fluroxypyr treatment. These results suggested that fluroxypyr destroyed the chloroplast structure of

299   foxtail millet, increases the risk of photo-oxidation damage, and reduced light absorption, transmission, and

300   distribution between PSII and PSI (Havaux, Strasser & Creppin, 1991).

301   Chlorophyll and gas exchange measurements are nondestructive methods used to identify the modes of

302   action of certain herbicides in the ecological risk assessment of herbicide exposure. In this study, chlorophyll

303   fluorescence showed that fluroxypyr treatment affected photosynthesis efficiency, even in the absence of

304   phenotypic symptoms (Qian et al., 2014). Our analyses showed that each fluroxypyr treatment caused different

305   degrees of F_v/F_m decrease, and the differences among treatments after 5 days of exposure were insignificant.

306   Compared with the control, only the maximum fluroxypyr dose (4-fold the recommended dose) showed

307   significantly reduced F_v/F_m values. Given that fluroxypyr inhibited electron transport in Zhangza 5, it also

308   caused the decline in Y(II) and ETR (II). Y(NPQ) represents the regulated photo-protective NPQ mechanism,

309   namely, dissipating the fraction of energy in the form of heat (Christof & Ulrich, 2008). Y(NPQ) also

310   dissipates excess PSII energy via the xanthophyll cycle and associated carotenoids (Deng et al., 2013). In the

311   present study, fluroxypyr treatment caused the increase in Y(NO), which demonstrated that the plant cannot

312   protect itself against damage from excess illumination due to blocking the regulatory mechanism of the non-

313   photochemical dissipation of energy. Consistent with our results, Guo et al. (2018) reported that sethoxydim

314   has similar effects on the photosynthetic pigments, photosynthetic gas exchange and chlorophyll fluorescence

315   parameters of foxtail millet.

316   Previous studies on several plant species have demonstrated that a typical feature of PSI photoinhibition

317   is the reduction in the maximum oxidation reduction ability of PSI (Scheller & Haldrup, 2005). In the present

318   study, Pm, Y(I), and ETR(II) decreased with increasing fluroxypyr doses, and were significantly inhibited

319   compared with those of the control. Fluroxypyr inhibited electron transport in Zhangza 5, which caused an

320   increase in Y(NA). Y(NA) is an important photo-damage indicator in PSI, and it is affected by dark adaptation

321   and level of damage to CO_2 fixation. Y(NA) was significantly increased by treatment with> 2 L ai ha^{-1}

322   fluroxypyr dose, showing that fluroxypyr aggravated the injury of PSII in foxtail millet leaves, reduced

323   electronic accumulation of acceptor-side in PSI, blocked the dark reaction process, and decreased the fixed

324   amount of CO_2. Y (ND) reflected the state of electron donors in PSI and is affected by the transmembrane

325   proton gradient and degree of damage of PSII (Yuan et al., 2013). In our study, Y(ND) in Zhangza 5 increased

326   at the fluroxypyr concentrations of ≤ 1 L ai ha^{-1}.

327   To further understand the mechanism of fluroxypyr toxicity about Zhangza 5, we analyzed the effect of

328   fluroxypyr on endogenous hormones. Herbicides induced changes in endogenous hormones and regulated

329   stomatal behavior. Zhou et al. (2014) reported that the stomatal behavior of plant leaves is related not only to

330   their content of endogenous hormones, but also to the balance of various hormones. Wang, Zhou & Zhou

331   (1994) reported that stomatal closure and transpiration reduction are affected by ABA, and also result from the

332   combined action of ABA and ZR. In the present study, the IAA and ABA contents in the plant leaves were

333   significantly increased; however, the GA and ZR contents significantly decreased. In our study, G_s values

334   showed a progressive decrease with the increase in fluroxypyr concentrations, while ABA content gradually

335   accumulated with the increase in fluroxypyr concentrations, implying that ABA inhibited photosynthesis by

336   decreasing stomatal conductance. In addition to stomatal regulation, ABA and ZR also directly influenced

337 photosynthesis. ABA reduced the electric potential of the chloroplast cell membrane, thereby reducing

338 photosynthetic electron transport, whereas ZR could increase the Rubisco activity and promote photosynthetic

339 electron transport.

340   High efficiency and safety are the main objectives of herbicide application. Eleven simplified indicators

341 were screened by Fisher discriminant analysis, and principal component analysis was used to comprehensively

342 evaluate the growth parameters, peroxidation characteristics, photosynthetic characteristics and endogenous

343 hormones of foxtail millet at different fluroxypyr dosages. The model was set up as follows: $F = 0.592Z_1 +$

344 $0.345Z_2$. The cumulative contribution rate of the first two principal components reached 93.72%. The first

345 principal component explained 59.23% of the variance including photosynthetic characteristics and

346 endogenous hormones of foxtail millet. The second principal component accounted for 34.49% of the variance

347 and had high loadings for growth parameters. Consequently, these two principal components were selected to

348 represent the comprehensive effect of fluroxypyr on foxtail millet.

349 **CONCLUSIONS**

350   The effect of fluroxypyr on foxtail millet is complex and involves combination of several factors. The

351 application of 1 L ai ha^{-1} fluroxypyr exerted minimal effects on growth parameters, oxidase activity,

352 photosynthetic activity, and endogenous hormones, indicating its efficient and safe benefits in foxtail millet

353 application.

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Figure 1

Figure 1. Effect of fluroxypyr on superoxide, peroxidase, catalase, ascorbate peroxidase, and glutathione reductase activities of foxtail millet.

SOD, POD, CAT, APX and GR represent superoxide, peroxidase, catalase, ascorbate

peroxidase, and glutathione reductase activities, respectively.
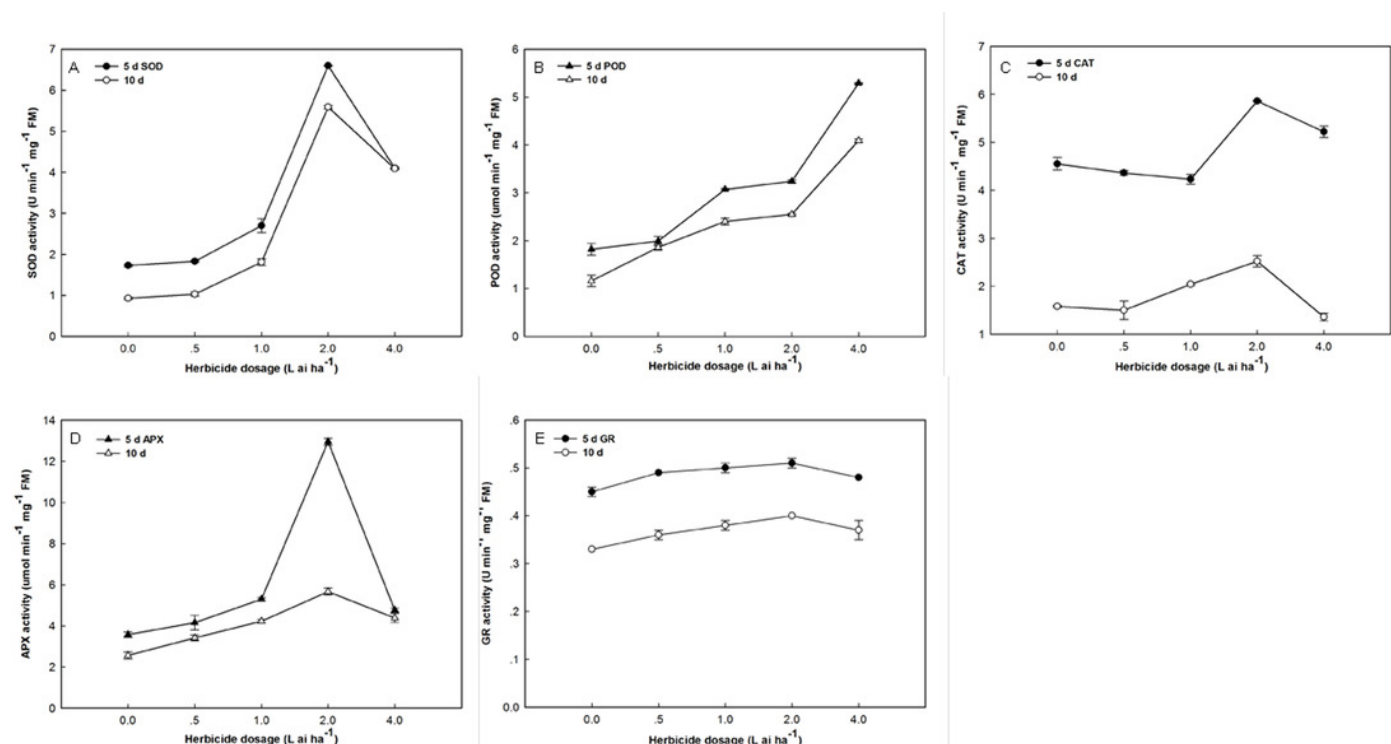
Figure 2

Figure 2. Effect of fluroxypyr on chlorophyll fluorescence and P_{700} parameters of foxtail millet.

F_v/F_m – maximum quantum yield of PS; Y(II)– PSII effective quantum yield; ETR(II)– PSII electron transport rate. Y(NO)– quantum yield of nonregulated energy dissipation in PSII; Y(NPQ)– quantum yield of regulated energy dissipation in PSII. Pm – maximal P_{700} change; Y(I)– photochemical quantum yield of PSI; ETR(I)– PSI electron transport rate; Y(NA)– quantum yield of nonphotochemical energy dissipation due to acceptor-side limitation in PSI; Y(ND)– quantum yield of nonphotochemical energy dissipation due to donor-side limitation in PSI.
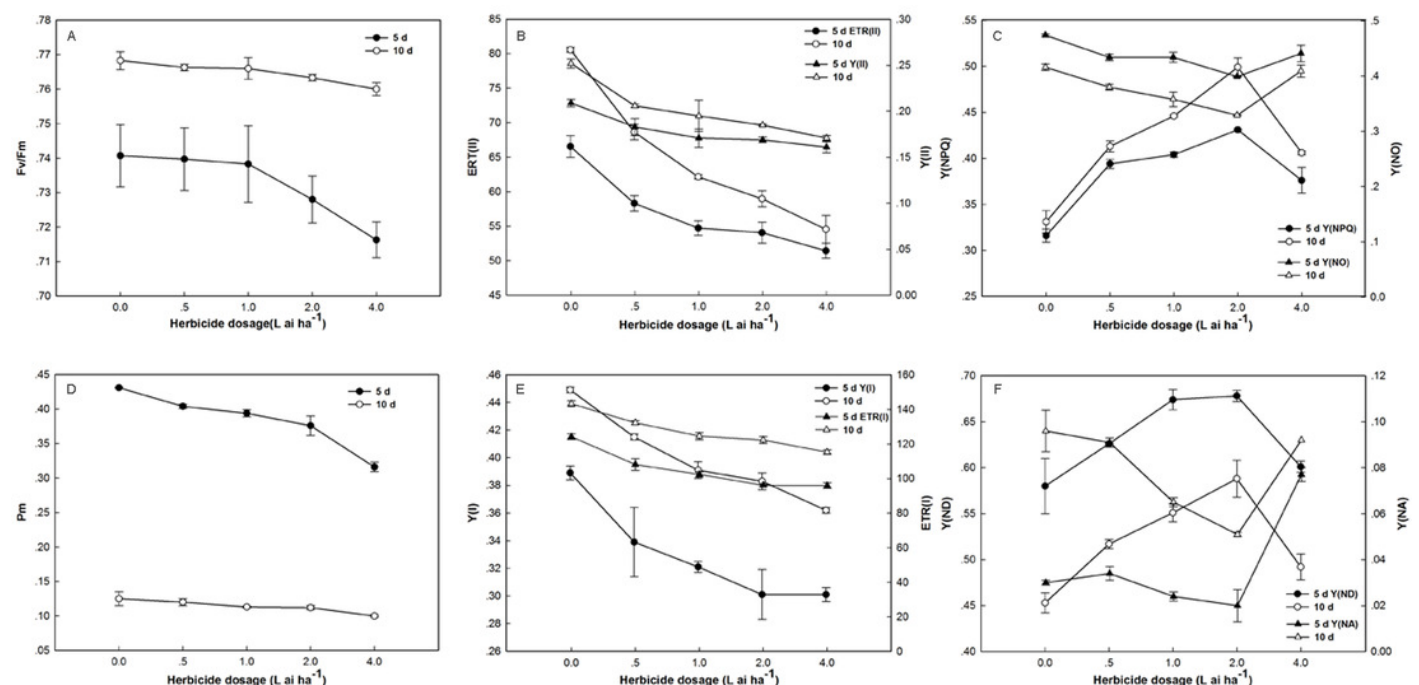
Figure 3

Figure 3. Effect of fluroxypyr on endogenous hormones of foxtail millet.

IAA, GA, ABA and ZR represent auxin, gibberellin, zeatin, abscisic acid, respectively.
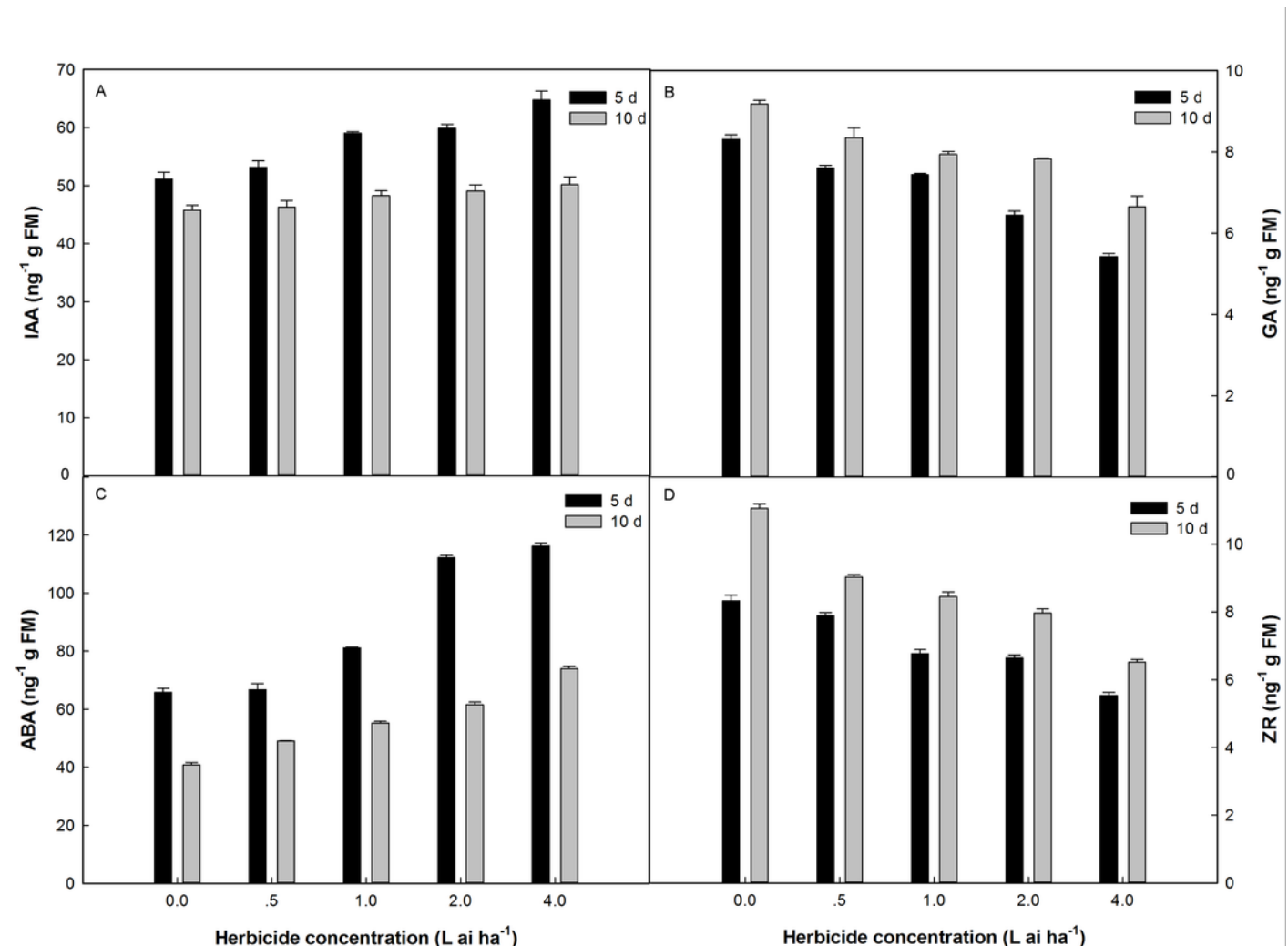
Figure 4

Figure 4. PCA score plot, the percentages of PC1 and PC2 representing the total variances of samples.

((1)-(3)) represent fluroxypyr dosage 4.0 L ai ha^{-1}, ((4)-(6)) represent fluroxypyr dosage 2.0 L ai ha^{-1}, ((7)-(9)) represent 0.5 L ai ha^{-1}, ((10)-(12)) represent fluroxypyr dosage 1.0 L ai ha^{-4}, and ((13)-(14)) represent fluroxypyr dosage 0 L ai ha^{-1} , respectively.
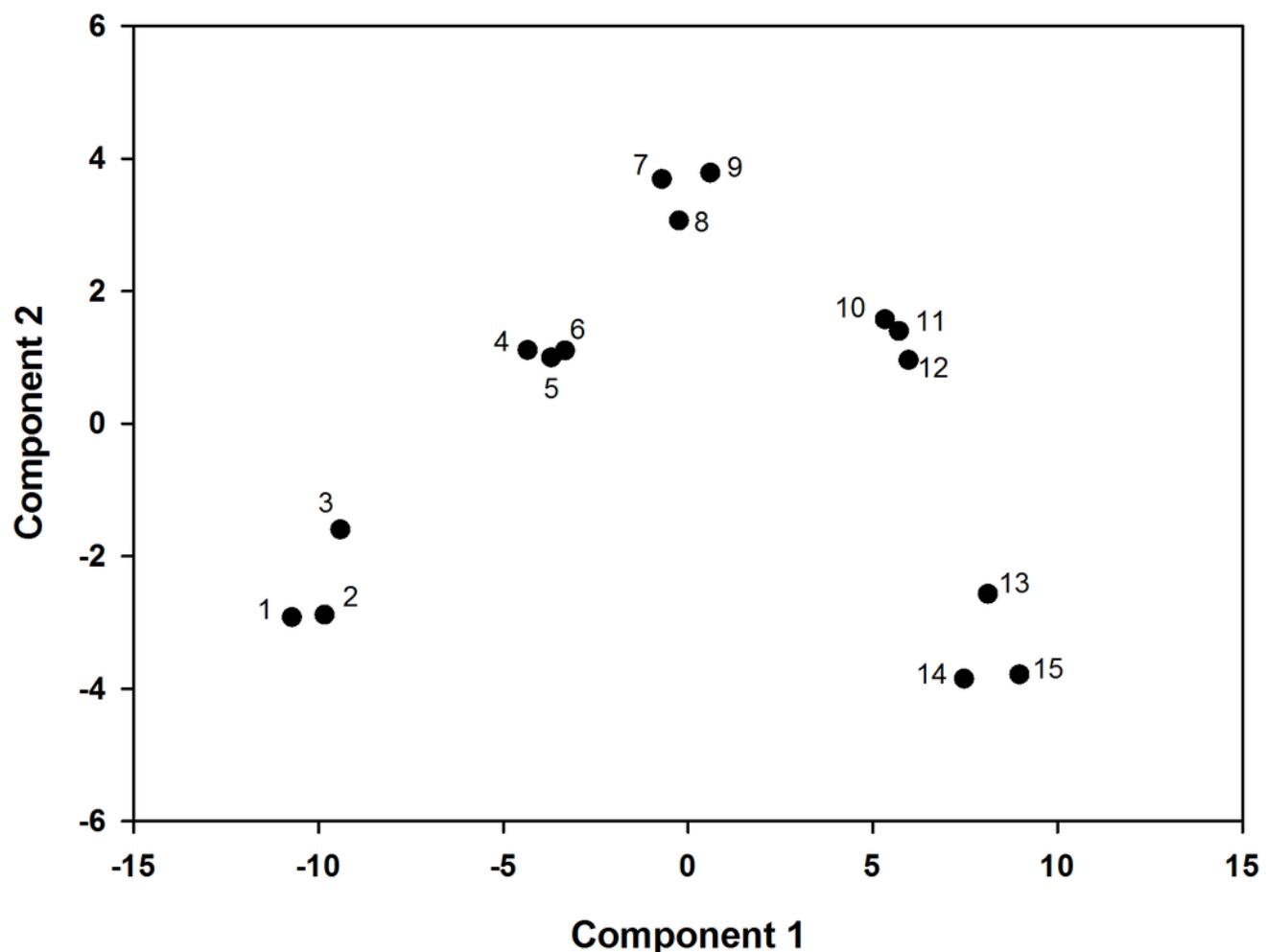
Table 1 Effect of fluroxypyr application on growth parameters in foxtail millet

Different letters in the same column indicate a significantly difference at the *P<0.05* level by Duncan's new multiple range test.

Table 1 Effect of fluroxypyr application on growth parameters in foxtail millet

Herbicide dosage	Plant height (cm)		Leaf area (cm^2)	
(L ai ha^{-1})	5 Day	10 Day	5 Day	10 Day
0	26.05±0.23c	30.50±0.34cd	8.00±0.48b	12.10±0.15b
0.5	29.95±0.46b	33.60±0.57ab	9.71±0.07b	12.50±0.18b
1	32.50±0.02a	34.35±0.23a	13.94±0.43a	15.49±0.32a
2	28.60±1.41b	32.25±0.95bc	8.70±0.69b	12.18±0.36b
4	23.60±1.21d	30.10±0.02d	7.78±1.77b	10.75±0.33c

2   Different letters in the same column indicate a significantly difference at the *P<0.05* level by Duncan's new

3   multiple range test.

Table 2 Effect of fluroxypyr application on stress parameters in foxtail millet

Different letters in the same column indicate a significantly difference at the $P<0.05$ level by Duncan's new multiple range test.

FM, fresh mass.

1 **Table 2 Effect of fluroxypyr application on stress parameters in foxtail millet**

Herbicide dosage (L ai ha^{-1})	H$_2$O$_2$ content (umol g^{-1} FM)		O$_2^-$ generating rate (nmol g^{-1} min^{-1} FM)	
	5 Day	10 Day	5 Day	10 Day
0	40.70±0.02c	15.53±0.05e	164.18±1.09d	153.06±0.41c
0.5	40.83±0.02c	23.69±0.03d	167.26±0.34c	159.45±0.95b
1	41.16±0.03c	31.40±0.05c	168.20±0.54c	160.87±0.95b
2	42.96±0.01b	36.40±0.06b	177.19±0.85b	162.05±0.54b
4	51.30±0.03a	39.06±0.07a	193.28±0.41a	166.07±1.63a

2 Different letters in the same column indicate a significantly difference at the *P<0.05* level by Duncan's new

3 multiple range test.

4 FM, fresh mass.

Table 3 Effect of fluroxypyr application on photosynthetic parameter and pigments in leaves of foxtail millet

Different letters in the same column indicate a significantly difference at the $P<0.05$ level by Duncan's new multiple range test. P_N, E, G_s and Chl represent photosynthetic rate,

Transpiration rate, stomatal conductance, chorophyll content, respectively.

1   **Table 3 Effect of fluroxypyr application on photosynthetic parameter and pigments in leaves of foxtail millet**

Herbicide dosage (L ai ha^{-1})	P_N[umol(CO$_2$)m^{-2}s^{-1}]		E[umol(H$_2$O$_2$)m^{-2}s^{-1}]		G_s[umol(CO$_2$)m^{-2}]		Chl(mg g^{-1})	
	5 Day	10 Day	5 Day	10 Day	5 Day	10 Day	5 Day	10 Day
0	16.86±0.31a	26.56±0.14a	2.57±0.01a	4.76±0.09a	67.53±0.18a	120.99±2.76 a	9.57±1.13a	14.24±0.75 a
0.5	14.81±0.52b	23.81±0.73 b	2.17±0.06a b	4.31±0.02 b	61.81±2.09b	108.81±0.95 b	9.26±0.03a	13.82±0.24 a
1	12.86±0.48c	21.57±0.91 b	1.99±0.32b	4.04±0.01c	65.14±0.79a b	98.25±2.63c	8.33±0.50a b	13.55±0.09 a
2	11.88±0.47c d	22.27±1.13 b	1.72±0.09b c	3.85±0.10c	51.67±1.93c	91.68±4.74c	7.17±0.07b c	11.75±0.24 b
4	11.28±0.47d	16.95±0.36c	1.24±0.04c	3.16±0.03 d	50.03±1.63c	74.71±2.55d	5.83±0.21c	10.92±0.42 b

2   Different letters in the same column indicate a significantly difference at the *P<0.05* level by Duncan's new multiple range test.

3   P_N, E, G_s and *Chl* represent photosynthetic rate, Transpiration rate, stomatal conductance, chorophyll content, respectively.

Table 4 Component matrix and variance explained by principal component

1 **Table 4 Component matrix and variance explained by principal component**

Traits	Component weight	
	Component 1	Component 2
Plant height	0.189	0.975
Leaf area	0.146	0.888
SOD activity	0.175	0.965
POD activity	-0.926	-0.245
GR activity	-0.702	0.640
Y(II)	0.963	-0.233
Y(NPQ)	-0.652	0.653
P_m	0.944	0.255
ETR(I)	0.978	-0.206
GA	0.975	0.184
ABA	-0.963	-0.178
Eigenvalue	6.515	3.794
Cumulative contribution (%)	59.230	93.722

Table 5 Principal score and general score of comprehensive evaluation

Table 5 Principal score and general score of comprehensive evaluation

Treatment	Prin1 score	Prin2 score	Composite score F
0.5	0.083	2.585	0.941
1	2.998	0.672	2.007
2	-4.242	0.638	-2.291
4	-7.850	-2.110	-5.375