

Manganese Stress in Flue-cured Tobacco: Biochemical Responses

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Research article

Keywords: Manganese toxicity, browning tobacco leaves, antioxidative systems, economic value, Yunnan

Posted Date: October 22nd, 2019

DOI: <https://doi.org/10.21203/rs.2.16312/v1>

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Abstract

Background: Manganese (Mn) stress is an important reason for forming tobacco (*Nicotiana tabacum* L.) leaf browning, leading to low yield and quality of tobacco leaves. The mechanism of flue-cured tobacco varieties, Honghuadajinyuan (Hongda) and K326 tobacco leaves browning by Mn were explored. The two tobacco varieties were planted in red soil and sandy soil and Mn concentration of 0, 250, 500, 750 and 1000 mg/kg were applied. The research investigate the traits of agronomic, physiological and economic of flue-cured tobacco.

Results: As Mn application rate increased, browning scale increased while plant height, root, stem and total weight, content of chlorophyll decreased. Activities of superoxide dismutase (SOD), catalase (CAT), and ascorbate peroxidase (APX) showed a rising parabolic formation. As Mn application rate increased, the content of malondialdehyde (MDA) and relative conductivity increased. Peroxidase (POD) activity continually increased with Mn application rate in K326 variety, while in Hongda variety, POD activity first increased and then decreased in sandy soil, while it continuously rose in red soil. Mn concentration and browning scale were significantly and negatively correlated with height, soil and plant analysis development (SPAD), CAT, total weight, average price and yield, but significantly positively correlated with total plant Mn content, POD and MDA. Plant Mn concentration was significantly and positively correlated with browning scale. This study founded that SOD, CAT, APX, POD enzyme activity and MDA content in tobacco leaves affected by Mn concentration in soils, cause the change of chlorophyll content, agronomic and economic traits of flue-cured tobaccos.

Conclusion: The mechanism of Mn stress to the physio-biochemical of tobacco varieties would be useful for controlling browning tobacco leaves by agronomic strategies and regarding as markers for Mn tolerance in breeding.

1 Background

Flue-cured tobacco in Yunnan province (about 3.33×10^5 ha per year by over 1,000,000 growers) yields 8×10^8 kg of leaves - approximately one half of the total production in China [1]. During growth browning in tobacco leaves generally causes dysplasia and inhibits photosynthesis, and at the flue-curing process seriously reduces the quality and yield [2,3]. One reason for browning tobacco leaves could be the weather, low temperature in tobacco growing later period is prone to form browning tobacco leaves. Besides, excessive manganese (Mn^{2+}) in field soils is another important reason for forming browning tobacco leaves. Planting mainly occurs in paddy soils in which the major preceding crop is rice (*Oryza sativa* L.) [4]. Long-term flooded cultivation in paddy soils leads to puddling, low pH, and low permeability resulting in excessive soluble Mn^{2+} in soil solution [5-7]. Plants growing in such paddy soils absorb Mn^{2+} to toxic levels [8]. However, the mechanism and physio-biochemical responses of tobacco leaf browning caused by excessive Mn^{2+} are still unclear.

Manganese is a necessary trace element and plays an irreplaceable role in photosynthetic oxygen evolution, maintaining normal organelle structure, and activating plant enzyme activity [9,10]. Three kinds of plant-available Mn occur in soils: water-soluble Mn, exchangeable Mn, and reducible Mn [11]. The first two occur in the form of Mn^{2+} , while the last refers to high-valency Mn oxides easily reduced to available Mn^{2+} [12]. Tobacco actively absorbs available Mn in soils to synthesize enzymes and chlorophyll needed for growth and development, promoting nitrogen metabolism, and regulating plant growth and phenolic metabolites [13,14].

Depending on the type of enzyme, the concentration of exogenous metal, and plant growth stage, there are different effects (promotion or inhibition) on enzyme activity [15-17]. When metal ion enters plants, they can bind to active or inactive sites on biomolecules, which changes physiological and metabolic functions, causing poisoning and even plant death [18]. As plants suffer from stress, the balance between the active oxygen species generated in metabolism and scavenging systems is affected [19,20]. Osmotic adjustment substances (i.e. proline and betaine) influence the generation and elimination of active oxygen species [21]. Detecting the changing external morphology of plants, integrity of the membrane system in plants, and enzyme activity generated in osmotic adjustment can indirectly reveal changes of physiological and biochemical reactions of plants in stress conditions [22].

There are relevant reports on Mn toxicity in tobacco [23-25]. The typical symptoms of Mn^{2+} toxicity in tobacco leaves are dark brown spots with chlorosis appearing on the tip and edge of old leaves, after which leaves wither [26,27]. Crinkle disease and chlorosis tend to happen in young leaves [28,29]. The white callus of tobacco leaf turns brown due to a pigment named protoporphyrin IX accumulation [26]. The correlation between the occurrence of browning tobacco leaves and critical Mn levels and the physiological mechanisms by which Mn stresses tobacco are still unclear, and is a restrictive factor for improving yield and quality in such situations [30]. Comparative research on Mn toxicity for the two main flue-cured tobacco varieties and interaction of different soils have not been reported. Based on reported stress effects of Mn, this research investigated and determined external morphologies and internal

physiological indices of poisoned plants for two different flue-cured tobacco varieties, soil types, and their interactions. Thus, we hypothesize that with increasing Mn concentration, the stress enzyme activity in tobacco leaves changes, leading to altered physiological metabolism of tobacco leaves, death, and browning. This will provide theoretical and production support for improving yield and quality of tobacco under Mn stress conditions.

2 Results

2.1 Effect of Manganese to agronomic traits, chlorophyll content and browning scale indices of tobacco leaves

Manganese application rate in soils had significant influence on agronomic traits, SPAD value, and browning scale of the tobacco plants. Variance analysis demonstrated that variety, Mn application rate, and soil type significantly influenced plant parameters such as height, root weight, stem weight, and total weight. There were also significant interactions of variety \times soil. Mn application rate for plant height and variety \times soil interactions for mass measurements (Table 1) ($P < 0.05$). Variety, soil, and Mn application rate also had significant impacts on SPAD whereas browning scale was only significantly affected by variety, Mn application rate, and the interaction of variety \times Mn application rate (Table 1).

Table 1 Variance analysis of effects of variety, soil type, and Mn application rates on agronomic indices

Source of variance	Degree of freedom	Plant height	Browning scale	SPAD value	Root weight	Stem weight	Total weight
Variety	1	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	0.0013
Soil	1	<0.0001	0.4035	<0.0001	<0.0001	0.0018	<0.0001
Concentration	4	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001
Variety \times Soil	1	<0.0001	<0.0001	0.2438	0.0014	<0.0001	<0.0001
Variety \times Concentration	4	0.0951	<0.0001	0.6457	0.1296	0.084	0.4968
Soil \times Concentration	4	0.0542	0.0084	0.5088	0.2652	0.000	0.4968
Variety \times soil \times Concentration	4	0.0435	0.0002	0.3519	0.5909	0.1353	0.3623

There was a significant interaction of soil \times Mn application rate for plant height in the two varieties (Fig. 1). As Mn application rate increased in sandy soil there were no significant differences between Hongda and K326 in terms of plant height. However, in red soil, Hongda plant heights were significantly higher than those of K326 (Fig. 1).

In the two soil types as Mn application rate increased, root, stem and total weight of the two varieties decreased (Fig. 1) and the effect was more pronounced in red soil. In sandy soil, the total weight of the two varieties had basically the same changes with Mn application rate, while the total weights of Hongda in red soil were higher than those of K326 at any Mn application rate. When concentrations of applied Mn were 0, 250 and < 500 mg/kg, the total weights of K326 variety in sandy soil were significantly larger than in red soil.

Variety, Mn application rate, and soil type all significantly influenced the chlorophyll content (SPAD value). As Mn application rate increased the chlorophyll content (SPAD value) in both varieties significantly decreased (Fig. 1). In different soils, no significant difference was observed in SPAD for the two varieties with different concentrations of applied Mn. In each soil type, SPAD values of Hongda were larger than those of K326 and SPAD values in red soil were larger (but not significantly so) than those in sandy soil with different Mn application rates.

When the Mn application rate was ≥ 500 mg/kg, the browning scales greatly increased in both soil types (Fig. 1). The browning scales of K326 with different Mn application rates were greater than those of Hongda. When Mn application rate was ≥ 500 (mg/kg) in red soil, browning scale of K326 was significantly greater than that of Hongda. The browning scales of Hongda at each Mn application rate in red soil were less than those in sandy soil and significantly lower when Mn application rates were 750 and 1000 mg/kg.

Figure 1. Various agronomic indices of variety, soil, and Mn application rate; Different letters represent statistical differences in the same soil types; stars above letters indicate significant difference between soil types.

2.2 Manganese concentration in tissue

Manganese application rate in soils had significant effects on Mn contents in tobacco roots, stems, and leaves (Table 2).

Table 2 Variance analysis of impacts of variety, soil type, and Mn application rates on contents of Mn in each part.

Source of variance	Degree of freedom	Root	Stem	Lower tobacco leaf	Middle tobacco leaf	Upper tobacco leaf
Variety	1	0.8871	0.0004	0.0257	<0.0001	0.6119
Soil	1	0.0023	0.1424	0.2652	<0.0001	0.0039
Concentration	4	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001
Variety × Soil	1	0.4423	0.0004	0.0751	0.0347	0.778
Variety × Concentration	4	0.3122	0.0169	0.648	0.0025	0.2591
Soil × Concentration	4	0.0161	0.0032	0.082	0.0553	0.164
Variety × soil × Concentration	4	0.3405	0.0159	0.0681	0.1991	0.4209

Variance analysis demonstrated that soil, Mn application rate in soil and their interaction significantly affected the Mn content in roots. Manganese concentrations in stems was greatly affected by variety, Mn application rate in soil and the interaction of variety × soil × Mn application rate. The Mn content in upper tobacco leaves was significantly influenced by soil and Mn application rate in soil. For middle tobacco leaves, variety, soil, Mn application rate in soil and the interaction of variety × soil and variety × Mn application rate in soil had great influences on Mn content. Manganese content in the lower tobacco leaves was significantly affected by variety and Mn application rate in soil.

The Mn concentration in roots increased with increasing Mn application rate (Fig. 2). When the Mn application rate was 1000 mg/kg, the root Mn concentration in sandy soil increased more obviously than that in red soil. The Mn concentration in the Hongda stems in sandy soil was significantly higher than that in red soil when 750 mg/kg and 1000 mg/kg Mn were applied. When the Mn application rate was 1000 mg/kg in sandy soil, the Mn concentration in Hongda stems was significantly higher than that in K326, while the former was slightly higher than the latter at other concentrations. The two varieties in red soil with different Mn application rates were not significantly different.

The Mn concentrations in the upper, middle, and lower tobacco leaves rose with increased Mn application rate. (Fig. 2). The Mn concentration in the upper tobacco leaves of K326 in sandy soil reached a maximum when the Mn application rate was 1000 mg/kg, while that in red soil was largest when the Mn application rate was 750 mg/kg. It can be seen from panels 7 and 8 in Fig. 2 that in both soils the Mn concentration in the middle leaves of Hongda were higher than those of K326. The Mn concentrations in Hongda stems in red soil were slightly higher than that in sandy soil. Moreover, when the Mn application rate was 250 mg/kg, Mn concentration in Hongda in red soil was significantly higher than that in sandy soil.

Figure 2. Manganese concentration in various above ground plant parts as a function of variety, soil, and Mn application rate; Different letters indicate statistical differences within the same soil types; stars above letters indicate significant difference between soil types

2.3 Oxidoreductase activity and relative conductivity

The Mn application rate exerted significant effects on oxidoreductase activity and relative conductivity of plants.

The SOD, POD, CAT, MDA, and relative conductivity were significantly affected by variety, soil, Mn application rate, and their interactions ($P<0.05$) (Table 3). The APX was affected by variety and Mn application rate, and their interactions.

Table 3 Variance analysis of influences of variety, soil type and Mn application rates on oxidoreductase activity and relative conductivity of tobacco leaves

Source of variance	Degree of freedom	SOD	POD	CAT	APX	MDA	Relative conductivity
Variety	1	<0.0001	<0.0001	<0.0001	0.0299	0.0653	<0.0001
Soil	1	0.0134	0.002	<0.0001	0.1117	0.0047	<0.0001
Concentration	4	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001
Variety × Soil	1	0.0015	0.3708	0.0345	0.0096	0.0084	0.0838
Variety × Concentration	4	<0.0001	0.2051	<0.0001	0.2193	0.1754	0.0008
Soil × Concentration	4	0.5203	0.1093	<0.0001	0.4897	0.0063	0.0081
Variety × soil × Concentration	4	0.6691	0.0776	<0.0001	0.1813	0.002	0.7953

Activities of SOD, CAT, and APX increased and then decreased with increasing Mn application rate (Fig. 3). Hongda in both soils had the highest SOD activity when the Mn application rate was 500 mg/kg and SOD activities of Hongda in sandy soil were higher than those of K326 for the same Mn application rates. In red soil, when Mn application rates were 500 and 1000 mg/kg, SOD activities of Hongda were considerably greater than that of K326. When Mn application rate was 1000 mg/kg in both soils, SOD activities of K326 were significantly lower than those of the control group, while SOD activities of Hongda were slightly higher than those of the control group.

The CAT activity of Hongda in sandy soil was highest when the Mn application rate was 250 mg/kg. When the Mn application rate was ≥ 500 mg/kg, CAT activities were lower than those in the control group. In red soil, CAT activity of Hongda reached a maximum at a Mn application rate of 750 mg/kg while that of K326 was greatest with a Mn application rate of 500 mg/kg. When the Mn application rate was ≥ 750 mg/kg, CAT activity of K326 in red soil was significantly higher than that in sandy soil. For Hongda, at a Mn application rate of 750 and 1000 mg/kg, CAT activities of the variety in red soil were higher than those in sandy soil. In addition, in sandy soil, when Mn application rate was 1000 mg/kg, CAT activity of Hongda was significantly lower than that of the control group, while the difference was nonsignificant in red soil.

The APX activities of Hongda and K326 in sandy soil were highest at an application rate of 500 mg/kg. When the Mn application rate was 1000 mg/kg, APX activity was lower (but not significantly) than that of the control group, while APX activities of K326 at different Mn application rates were higher than those of the control group. In red soil, the APX activity of Hongda was highest with a Mn application rate of 750 mg/kg, while that of K326 reached a maximum when the Mn application rate was 500 mg/kg and then decreased drastically. Furthermore, when 1000 mg/kg of Mn was applied, the enzyme activity was greatly reduced compared with the highest value. The APX activities of the two varieties with different Mn application rate did not show significant differences in comparison with the control.

The POD activity was significantly affected by variety, soil and Mn application rate. POD activity of K326 in sandy soil gradually increased with increasing concentration of applied Mn and significantly rose compared with that of the control with a Mn application rate of 1000 mg/kg. POD activity of Hongda first increased then decreased with a maximum at a Mn application rate of 500 mg/kg. In red soil, POD activities of the two varieties gradually rose with Mn application rate and POD activity of K326 were typically higher than those of Hongda.

The MDA contents in the two varieties in sandy soil increased and then did not change and peaked at a 750 mg/kg Mn application rate. The MDA contents of Hongda with different applied concentrations rose insignificantly compared with those of the control, while MDA content in K326 increased greatly when Mn application rate was ≥ 750 mg/kg in comparison with the control. The MDA contents in the two varieties in red soil gradually rose and peaked when the Mn application rate was 1000 mg/kg. Moreover, MDA contents in K326 at different Mn application rates were higher than those in Hongda.

Relative conductivity showed basically the same change trend as MDA and gradually increased in these two varieties in both soils. In comparison with K326, the increment of relative conductivity of Hongda was greater. In sandy soil, when the Mn application rate was ≥ 750 mg/kg, relative conductivity of K326 was significantly different from the control. The change trend of relative conductivity of the K326 and Hongda varieties in red soil was similar with that in sandy soil. However, relative conductivities of Hongda in different Mn application rates were significantly different with the control, while those of K326 presented nonsignificant differences.

Figure 3 Oxidoreductase activity and relative conductivity of tobacco leaves under conditions of different variety, soil, and Mn application rate; Different letters indicate statistical differences in the same soil types; stars above letters indicate significant difference between soil types

2.4 Economic traits

The Mn application rates in soils had significant effects on the economic traits of tobacco leaves.

The weight of the lower and middle tobacco leaves was significantly affected by variety, Mn application rate and their interactions ($P<0.05$) (Table 4). The weight of upper tobacco leaves was greatly influenced only by variety and Mn application rate. Variety, soil, Mn application rate and their interactions had significant influences on yield as well as had remarkable effects on average price.

Table 4 Variance analysis of influences of variety, soil type and Mn application rates on various economic indices of tobacco leaves

Source of variance	Degree of freedom	Weight of lower leaf	Weight of middle leaf	Weight of upper leaf	Yield	Average price
Variety	1	0.0298	<0.0001	0.012	0.0057	0.2257
Soil	1	0.0784	<0.0001	0.843	<0.0001	<0.0001
Concentration	4	<0.0001	<0.0001	0.0002	<0.0001	<0.0001
Variety × soil	1	0.0635	<0.0001	0.7933	0.0012	0.0015
Variety × concentration	4	<0.0001	0.5758	0.3342	0.6515	0.0263
Soil × concentration	4	<0.0001	0.125	0.789	0.015	0.0189
Variety × soil × concentration	4	0.0006	0.445	0.6731	0.0604	0.1216

The weights of the upper tobacco leaves of the two varieties in sandy and red soil with different Mn application rates were lower than those of the control (Fig. 4). In the same type of soils, there were no significant differences in terms of weights of the upper tobacco leaves of the two varieties with different Mn application rates. The weights of the middle tobacco leaves declined in all treatments with increasing Mn application rate (Fig. 4). In sandy soil, the middle tobacco leaves weights of K326 were larger than those of Hongda. The weights of middle tobacco leaves of the two varieties in red soil were basically the same and differences were small. The weights of the lower tobacco leaves with different Mn application rates were basically the same, if slightly lower than those of the control (Fig. 4). Average prices of the K326 and Hongda varieties in the red and sandy soil gradually reduced with increasing Mn application rate and the average prices of the two varieties in each soil type with different Mn application rates were significantly lower than those in the controls group. When the Mn application rates were 250, 500, and 1000 mg/kg, the average prices of K326 in sandy soil were higher than those in red soil. Hongda in sandy soil showed significantly higher average price than in red soil only at the Mn application rate of 250 mg/kg, while the difference was insignificant in other conditions. When the Mn application rate was 1000 mg/kg, the average prices of the two varieties in the two types of soils decreased by about 50% compared with those in the control.

Figure 4 Various economic indices of tobacco leaves under conditions of different variety, soil, and Mn application rate; Different letters indicate statistical differences in the same soil types; stars above letters indicate significant difference between soil types

Table 5 Correlation analysis of agronomic, physiological, and economic indices with browning and Mn contents

	Height	SPAD	LWR	RC	TC	SOD	POD	CAT	APX	MDA	Tweight	Yield	Av
---Agronomic traits ---				----- Physiological indices -----						-----Economic traits-----			
Browning	-0.62962*	-0.77445*	0.12862	0.52306*	0.83788*	-0.1867	0.74867*	-0.29406*	-0.15451	0.8387*	-0.87412*	-0.60626*	-0.881
Mn contents	-0.46027*	-0.81901*	0.02427	0.71679*	0.94079*	-0.05517	0.73124*	-0.32302*	-0.01692	0.89907*	-0.86947*	-0.68347*	-0.951

Note: The values in the table are the correlation coefficients and * represents a significant correlation ($P<0.05$). LWR: leaf length and width ratio; RC: relative conductivity; TC: total Mn contents in plants; Tweight: total weight; Avprice: average price.

Table 5 demonstrates that Mn application rate and browning scale were significantly and negatively correlated with height, SPAD, CAT, total weight, average price and yield, but significantly and positively correlated with total Mn contents in plants, POD and MDA ($P<0.05$). Mn application rate was significantly and positively correlated with browning scale, and the relation between Mn and other enzymes had the same pattern with browning scale.

3 Discussion

3.1 Effect of Manganese to agronomic traits, chlorophyll content and browning scale indices of tobacco leaves

The increased of availability of Mn can affect the normal growth and yield of tobacco plants [26]. When Mn accumulates sufficiently in plants, growth is inhibited and toxicity symptoms occur, like short plants, chlorosis, and biomass decline. Studies show that excess Mn

reduces the CO₂ assimilation rate and stomatal conductance, which reduces the total biomass [31]. The decrease in agronomic traits with increase in Mn in this study showed that the Mn stress influenced the normal growth of tobacco plants and chlorophyll content in leaves, which was consistent with the results obtained by Wang et al [32]. Browning scale rose slowly with increasing Mn application rate, reducing the quality of tobacco leaves. The Hongda variety appears to tolerate Mn stress better than K326, which might be related to photosynthesis and its related chloroplastic proteins [9]. At the same Mn application rate and in the same soil, agronomic performance was higher and the browning scale of Hongda variety was lower than that of K326.

3.2 Accumulation of Mn in tobacco plants

Manganese accumulation was measured in roots, stems and leaves of two tobacco cultivars to examine their ability to tolerate Mn toxicity levels in different soil types. In this study, the Mn uptake in red soil was less than in sandy soil. The reason could be that the CEC and clay content in red soil was higher than sandy soil, which could absorb allowed more sorption of Mn²⁺ ion. Previous research indicates that the ability of plant to tolerate Mn toxicity is related to nutrients such as calcium (Ca) and magnesium (Mg), in ryegrass. Magnesium and Ca concentrations in all genotypes decreased concomitantly with increasing Mn applications [7]. Kováčik et al. [14] found different plant species have different trends of Mn accumulation; chamomile (*Matricaria chamomilia*), preferentially accumulated Mn in the roots compared to shoots, but a related species, *Tanacetum parthenium*, contained more Mn in shoots. In the present study, with the increase of Mn application rate, root weight and stem weight of the two varieties decreased. The two varieties showed a different result for accumulating Mn. For Hongda variety, the ability to tolerate Mn was obvious than that of K326 variety, since the Mn concentration in Hongda stems and middle leaves was higher than that in K326

3.3 Oxidoreductase activity and relative conductivity

Heavy metals can change the induction of enzymes active in oxygen scavenging systems and reduce the scavenging function of endogenous active oxygen species in plants [33,34]. This results in accumulation of many groups, such as O₂, H₂O₂ and OH⁻, which influences normal metabolic activity of cells, even producing stress effects [35]. The activities of three enzymes (i.e. SOD, CAT and APX) increased and then decreased with increasing Mn application rate. For plants with low-concentration Mn stress, to maintain normal physiological and circulatory metabolism, the activities of SOD and CAT enzymes increased, reducing the harm of the Mn stress [36]. However, when the concentration of applied Mn became excessive, the passively generated SOD and CAT enzymes in plants were unable to eliminate the peroxides generated due to stress [37,38]. In addition, the accumulation of Mn in plants decreased cell permeability and the activities of SOD and CAT enzymes, thus reducing enzyme activity.

SOD can eliminate harmful substances produced by organisms during metabolism [39]. SOD catalyzes disproportionation of superoxides and is the first antioxidant enzyme in an active oxygen scavenging system. Plants can produce active oxygen and free radicals that are harmful to cells in stress, and hydrogen peroxide and oxygen are generated by active oxygen through disproportionation of SOD, which protects cells and avoids or reduces harm from active oxygen [40]. When the Mn application rate increased, the activity of SOD enzyme in Hongda rose more than that of K326. This indicated that during stress, Hongda rapidly generated a lot of SOD, thus eliminating free radicals in plants and maintaining normal physiological metabolism of cells. By applying different Mn concentrations to cucumber seedlings, Zhou et al. [41] studied the effects of cold resistance of cucumber seedlings in response to increasing Mn. The results demonstrated that applying nutrient solutions containing Mn increased SOD activity of cucumber leaves, which raised cold resistance of cucumber seedlings to varying degrees, which is consistent with our results.

POD is an oxidoreductase generated by microorganisms or plants and plays a role in eliminating oxides in cells and preventing cell damages. Its activity reflects the adaptability of plants to adversity stress [42]. The oxidation of Mn (II) by a H₂O₂-consuming peroxidase (POD) has been proposed to be the key reaction leading to Mn toxicity symptoms, probably accompanied by the formation of reactive intermediate compounds like phenoxy radicals and Mn (III) [43]. POD activity in the leaf tissue increased with Mn treatment [44].

CAT is a protective enzyme showing anti-aging effects on organisms and can maintain stability and integrity of cell membranes [45]. The activity of CAT in K326 rapidly rose and then sharply decreased. The activity of CAT in Hongda slowly increased and then decreased. When the Mn application rate was 1000 mg/kg, the activities of CAT in Hongda in the red and sandy soils were higher than those in K326.

This trend has been observed in other plants, such as grapes [46]. The rapid CAT changes in K326 suggest greater sensitivity to Mn stress.

APX is an essential antioxidant enzymes that plants use to scavenge active oxygen and play an important role in the metabolism of ascorbic acid and removing H₂O₂ and hydroxyl radicals in plants [47]. Our results concur with the research results obtained by Shi et al [34]. This demonstrates that Hongda can better maintain the balance of microenvironment in the plant under the stress of high concentrations of Mn.

When plant organs senesce or are harmed in adversity, membrane lipid peroxidation generally occurs. MDA is one of the products of membrane lipid peroxidation and it's a reflection of the damage degree of plantsm[48]. When sandy soil was applied ≤ 500 mg/kg of Mn, the MDA content in Hongda was higher than that in K326. When Mn application rates were 750 and 1000 mg/kg, MDA contents in K326 significantly increased in comparison with the content at 500 mg/kg, while that of Hongda rose insignificantly. The absolute increment of MDA in Hongda was lower than that in K326. In red soil, MDA contents in K326 at the different Mn application rates were higher than those of Hongda. These results suggest that during Mn stress the amount of membrane lipid peroxidation in Hongda was lower than that of K326 at the higher Mn application rates. This phenomenon indicates that Hongda has stronger tolerant ability than K326.

Relative electric conductivity is an important physiological and biochemical index revealing the state of the membrane system of plants. When plants suffer from stress, the cell membrane easily fractures and membrane proteins are damaged, causing loss of cytosol, thus raising plant cell relative electric conductivity [49]. Conductivities of the two varieties in the two types of soils increased with increasing Mn application rate, indicating that both varieties were experiencing Mn stress with changing permeability of cell membranes. In the two soil types, the increase of relative conductivity of the two varieties in sandy soil was more obvious than that in red soil. Compared with sandy soil, red soil has a greater buffer ability to adsorb Mn. While this reduced the content of Mn available to plants in soil the permeability in red soil is poorer, which inhibited the migration of Mn in soils around roots.

3.4 Economic traits

The excessive content of Mn in soil not only reduces plant productivity and influences crop quality but also decreases yield and average price of tobacco leaves. Manganese stress had a greater influence on K326 than on Hongda and the effects of Mn stress on tobacco plants in sandy soil were greater than those in red soil. With increasing Mn application rate, the average prices and yields of tobacco leaves of the two varieties gradually decreased and reached a minimum when 1000 mg/kg of Mn was applied. Correlation analysis of agronomic, physiological, and economic indices revealed that Mn concentration in soils influenced oxidoreductase activity by adjusting the content of Mn in plants, thus changing agronomic traits and reducing economic traits.

4 Conclusions

Increasing Mn concentration was significantly and negatively correlated with CAT, but significantly and positively correlated with POD and MDA, leading to higher relative electrical conductivity and cell dell. Applied Mn was significantly and positively correlated with browning scale, resulting in the poor quality and lower yield. By increasing the concentration of applied Mn, the Mn content in roots and stems was increased more in sandy soil than red soil, yield and average price was reduced more significant for varieties planted in sandy soil. Therefore, compared with sandy soil, red soil is beneficial for maintaining soil environment and improving agronomic and physiological traits, yield and quality, at the meantime, reducing the damage of Mn stress to plants. With respect to variety, K326 are more easier to suffer the damage of Mn stress than Hongda.

5 Methods

5.1 Overview of the experiment environmental conditions

In Yunnan Academy of Tobacco Agricultural Sciences's Yanhe Research Farm near Yuxi, Yunnan, China (24°14'N 102°30'W) with an altitude of 1,680 m, this study was arrangemented. From March to September 2017 the annual average temperature and annual rainfall of the region are 15.9 °C and 918 mm, respectively with 2,072 h of annual sunshine. Rainfall from April to September accounts for 79.5%

of the annual precipitation. The actual precipitation and temperature conditions from March 1 to September 30 2017 are in Figure 5. The soil types (sandy soil and red soil) were used.

Figure 5 The precipitation and temperature conditions of study site from March 1st to September 30th 2017

5.2 Experiment design

Zhongyan Tobacco Seed Co., Ltd. provided two tobacco varieties, K326 and Hongda. Two varieties were cultivated and managed according to guidelines recommended by Yunnan Academy of Tobacco Agricultural Sciences. On March 5, using a floating system to conduct seedling production, and on May 4 transplanted the seedlings into prepared pots. On day 15 after topping (removal of flowers at the top of the plant), irrigated the roots for the first time and then irrigated every seven days (irrigated three times in total).

The experiment was conducted with plastic pots 36 cm in diameter and 28 cm high containing 20 kg of soil. One tobacco plant was planted per pot. The entire experiment after transplantation was carried out outdoors. Temperature and precipitation for the study period are in Fig. 5. The study investigated soil types (sandy soil and red soil) and cultivars (K326 and Hongda) by irrigation with Mn²⁺ (as MnSO₄ solution) at 0, 250, 500, 750 and 1000 mg/kg, continuously. A randomized block design was utilized in the experiment with each treatment replicated three times. Total experimental units were 60 (2 soil types*2 cultivars *5 concentrations *3 replicates). Red soil was classified as an Ultisol, based on the USDA soil taxonomy. [50]. Table 6 showed the basic nutrients of the two soil types. We used acetic acid (<2.5% w/v) to control soil pH value at 5.5 strictly once a week. Other agronomic management was similar for both soil types (e.g. nitrogen fertilizer management).

Table 6 Basic nutrients of the two soil types

Organic matter	Total nitrogen	Total phosphorus	Total potassium	Soil texture	CEC	Original soil Mn concentration.	Original soil pH value
		g·kg ⁻¹			(cmol/kg)	(mg/kg)	
15.36	1.04	0.33	6.37	28%sand+62%silt+10%clay	20.4	118.6	5.8
5.7	0.54	0.08	3.43	70%sand+23%silt+7%clay	5.9	67.3	6.0

5.3 Analyses and methods

5.3.1 Analyses and methods for agronomic indices

Plant height: The height of plants from stem base on the ground to the top of the stem was measured immediately before decapitation.

Dry mass: The dry weight of upper leaves, middle leaves, lower leaves, roots and stem weight was determined after killing at 105 °C and drying at 60 °C until mass was stable.

Soil and plant analysis development (SPAD) value: The SPAD value was determined by the SPAD-502 portable chlorophyll detector on August 20. Each treatment tested the middle part of the 6th leaf from the bottom to the top of the plant.

Browning scale: The degree of browning was divided into six levels. Level 0 indicates that the entire leaf was healthy. Level 1 indicated that browning and black spots were sporadically distributed on main or branch veins, accounting for no more than 5% of the leaf area. Levels 2, 3, and 4 had browning and black spots accounting for 5%~15%, 15%~30%, and 30%~45%, respectively of the leaf area. Level 5 indicated that browning and black spots occupied more than 45% of the leaf area. Level 6 denoted that browning and black spots occupied more than 45% of the leaf area, and these spots appear on petioles and spread to stems.

5.3.2 Analyses and methods of physiological indices

Compared with lower and upper leaves, mature middle leaves have the larger bio-mass and more serious Mn²⁺ toxicity symptoms, so mature middle leaves were used to evaluate the physiological indices

Determination of relative electrical conductivity: The conductivity method [51] was used as follows. The debris on the surface of fresh leaf samples was removed by running water gently and then washed twice with deionized water. The water on the surface of leaves was gently and completely absorbed by filter paper and 0.2 g of leaves was cut into strips in 5 mm widths. The strips were put into a 50 mL

test tube with a stopper and 20 mL of deionized water was added to immerse the samples for a 5 h incubation at room temperature. The conductivity (E1) was measured using a Mettler Toledo Five Easy conductance meter and the tubes placed in a boiling water bath for 15 min. After cooling to room temperature, the total conductivity (E2) was measured. The ratio of E1/E2 was used as relative conductivity to show permeability of the cytoplasmic membrane.

Determination of superoxide dismutase (SOD) activity: The SOD activity was determined by using the SOD-1-Y SOD kit produced by Suzhou Comin Biotechnology Co., Ltd. The unit was U/g (fresh weight) [52].

Determination of peroxidase (POD) activity: The POD-1-Y POD kit produced by Suzhou Comin Biotechnology Co., Ltd. was used to determine POD activity. The unit was U/g (fresh weight) [53].

Determination of CAT activity: The CAT activity was determined by employing the CAT -1-Y CAT kit manufactured by Suzhou Comin Biotechnology Co., Ltd., which followed the modified method of Beers and Sizer [54]. The unit was nmol/min/g (fresh weight).

Determination of ascorbate peroxidase (APX) activity: The determination of APX activity was conducted by using the APX-1-W APX kit from Suzhou Comin Biotechnology Co., Ltd. The unit was $\mu\text{mol}/\text{min}/\text{g}$ (fresh weight).

Determination of malonaldehyde (MDA) content: The MDA content was determined by using the MDA-1-Y MDA kit produced by Suzhou Comin Biotechnology Co., Ltd. The unit was nmol/g (fresh weight) [55].

Determination of total Mn in plant samples: The wet ashing technique (nitric and perchloric acid digestion method) and atomic absorption spectrophotometry were used in the determination [56].

5.3.3 Analyses and methods for indices of economic traits

The sum of the upper, middle, and lower dry tobacco leaves after the leaves reached maturity represented the total mass of tobacco leaves. The average price was determined based on *Standard of the People's Republic of China for Flue-cured Tobacco (GB2635-1992)* and calculated in accordance with procurement price lists of flue-cured tobaccos in 2017.

5.4 Statistical analysis

Data were analyzed with the General Linear Model (GLM) procedure of the SAS 9.3 computer package (SAS Institute Inc., Cary, NC). Significance of statistical analysis was based on a significant level of P value <0.05 . The average value was classified in the 95% confidence interval through Tukey's (HSD) test. Sigma Plot 12.3 (Systat Software Inc., Chicago, IL, USA) was used to create the figures.

Abbreviations

Mn: Manganese; SOD: superoxide dismutase; CAT: catalase; APX: ascorbate peroxidase; MDA: Malonaldehyde; POD: Peroxidase; Mn^{2+} : excessive manganese; LWR: leaf length and width ratio; RC: relative conductivity; TC: total Mn contents in plants; SPAD: Soil and plant analysis development

Declarations

Ethics approval and consent to participate

Not applicable

Consent for publication

Not applicable

Availability of data and materials

All data generated or analysed during this study are included in this published article.

Competing interests

Congming Zou and Yan Li and other co-authors have no conflict of interest.

Funding

This study was financially supported by several projects from the National Natural Science Foundation of China (No.41601330), Yunnan Science and Technology Innovation Project (2019HB068), Yunnan Ten Thousand People Program (2018-73), Yunnan Applied Basic Research Projects (No.2017FB074) and Tobacco Monopoly Bureau China (2016YN28, 2017YN09 and 2018530000241017).

Authors' contributions

Conceptualization, Congming Zou and Xiaopeng Deng, Wei Huang, Yi Chen; Data collection, Guorui Pu and Jiaen Su; Design of methodology, Yan Jin and Junying Li, Zhonglong Lin; Ke Ren and Yanjie Chen prepared figures 1-5 and edited the manuscript; Congming Zou and Yan Li wrote the main manuscript text.

Acknowledgements

The authors are thankful to M.S. Coyne for his valuable assistance and advice in the preparation of this paper.

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Figures

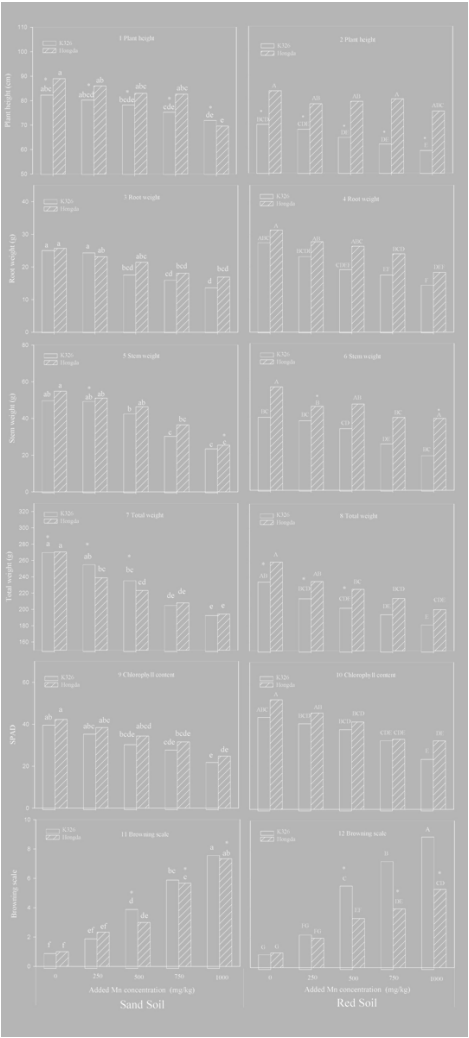


Figure 1
Various agronomic indices of variety, soil, and Mn application rate; Different letters represent statistical differences in the same soil types; stars above letters indicate significant difference between soil types.

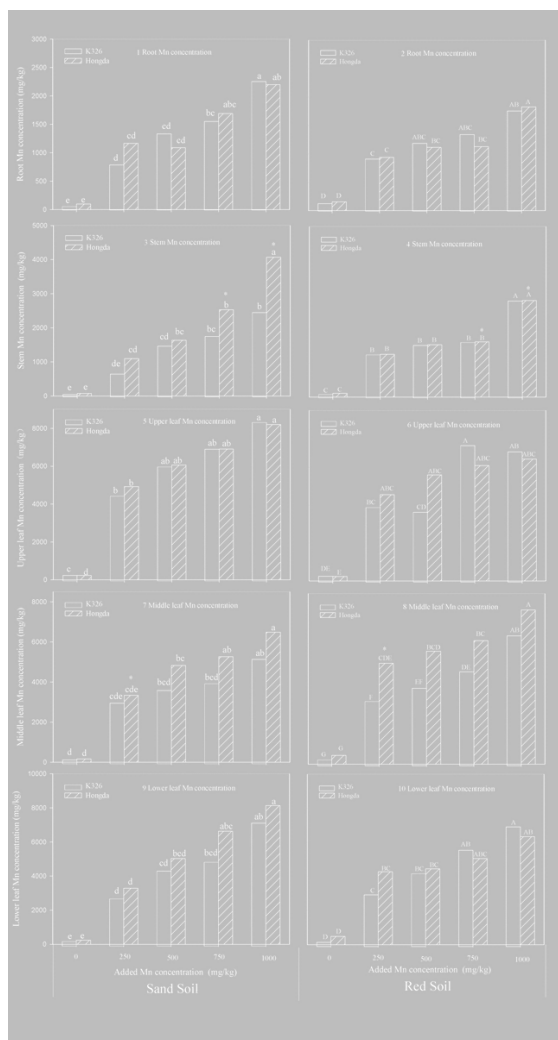


Figure 2

Manganese concentration in various above ground plant parts as a function of variety, soil, and Mn application rate; Different letters indicate statistical differences within the same soil types; stars above letters indicate significant difference between soil types

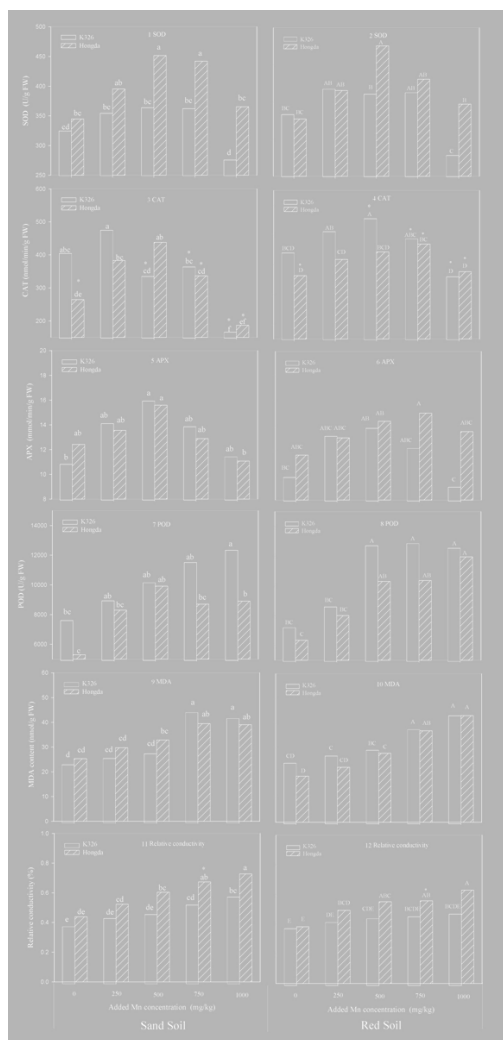


Figure 3

Oxidoreductase activity and relative conductivity of tobacco leaves under conditions of different variety, soil, and Mn application rate; Different letters indicate statistical differences in the same soil types; stars above letters indicate significant difference between soil types

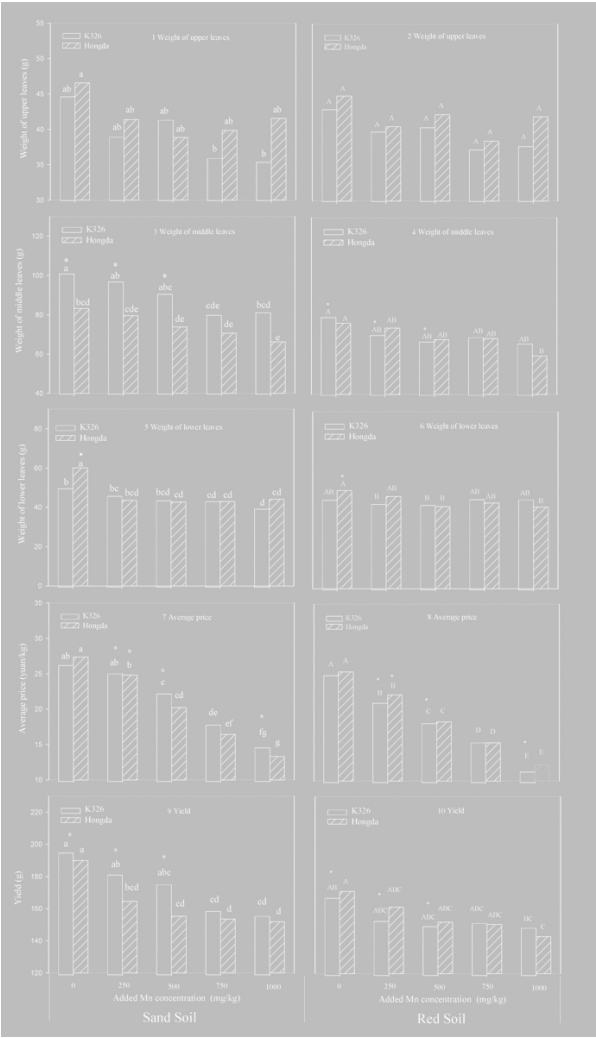


Figure 4

Various economic indices of tobacco leaves under conditions of different variety, soil, and Mn application rate; Different letters indicate statistical differences in the same soil types; stars above letters indicate significant difference between soil types

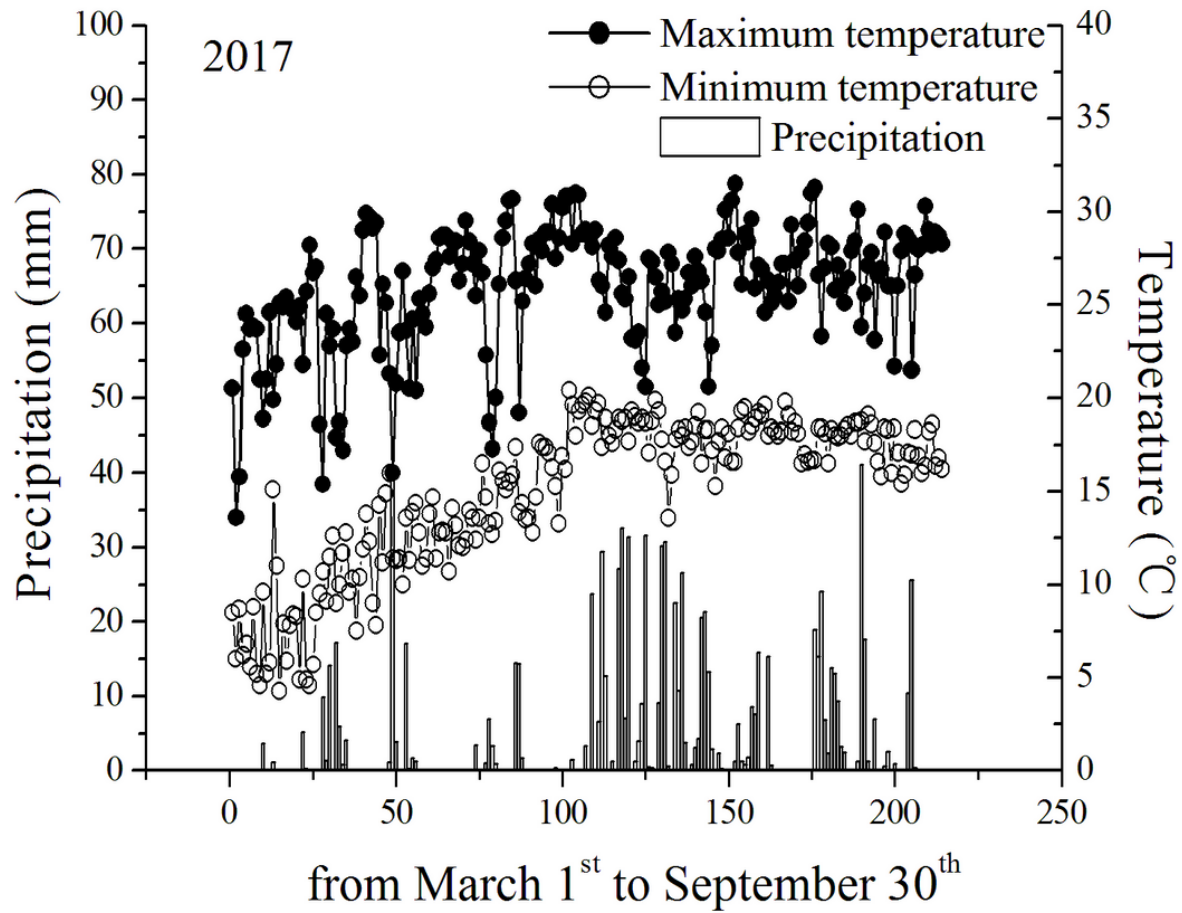


Figure 5

The precipitation and temperature conditions of study site from March 1st to September 30th 2017