***Pak. J. Bot.*, 51(4): 1349-1355, 2019.** **DOI: http://dx.doi.org/10.30848/PJB2019-4(35)**

LEAF IONOME TO PREDICT THE PHYSIOLOGICAL STATUS OF NITROGEN, PHOSPHORUS, AND POTASSIUM IN CAMELLIA OLEIFERA

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Abstract

The mineral nutrient and trace element composition of a tissue or organism is known as its ionome. In the present study, a statistical method to predict the physiological status of nitrogen (N), phosphorous (P), and potassium (K) using the leaf ionome in *Camellia oleifera* was explored. The latter is an important non-wood forest shrub for edible oil production in China. The elements N, P, K, calcium (Ca), magnesium (Mg), iron (Fe), manganese (Mn), boron (B), zinc (Zn), copper (Cu), and molybdenum (Mo) in the leaves of the sand-cultured seedlings with N, P, or K deficiency were investigated using Inductively coupled plasma mass spectrometry (ICP-MS). Significant relationships among the ions were found, and the leaf N, K, B, and Cu ions under N deficiency, the N, P, Fe, and Cu ions under P deficiency, and the N, K, B, Cu, Fe, and Mg ions under K deficiency showed obvious variations. Additionally, the principal component analysis (PCA) was introduced to reduce the dimensions of the multivariables based on the leaf ion concentrations. The PCA established that the leaf ionome contained information that could discriminate between the seedlings based on their N, P, or K status. Thus, statistical models were developed that could be used to classify the *C. oleifera* seedlings by their response to the N, P, and K deficiency or sufficiency, based on logistic regression. The area under the receiver-operator curves (AUC) showed that the P and K status prediction models built using the leaf ionome performed far better than those using single ions. Additionally, a >85% accuracy was obtained in discriminating between the seedlings under nutrient deficiency. The P prediction model also exhibited excellent specificity in the tests under N, K, Mg, or Mn deficiency.

Key words: *Camellia oleifera*, Leaf ionome, Nutrient deficiency, Multivariable logistic regression.

Introduction

Camellia oleifera, an important non-timber forest shrub, is mainly distributed in the hilly areas of southern China. Camellia oil, extracted from the *C. oleifera* seeds, has been reported to prevent hypertension, cardiovascular disease, and aging, as well as improve body immunity. It has been used in China for more than 1000 years (Zhuang, 2008; Cao *et al*., 2017). Of late, although the cultivation area of *C. oleifera* is up to 4 million hectares in China, the yield is severely limited due to the widespread shortage of N, P, and K in the acidic soil which has a pH between 4.5-6.5 (He *et al*., 1993; Zheng *et al*., 2010; Liu *et al*., 2017). Moreover, efficient nutrient management has become the focus in these areas, especially for P nutrition (Chen *et al*., 2010; He *et al*., 2011; Yuan *et al*., 2017). It is well known that the absorption and utilization of nutrients in plants are affected by their genes and the environment. The physiological status of these plants with reference to their nutrient concentrations is their response to the environment (Baxter, 2010). The plant under investigation in this study, *C. oleifera*, is rich in genetic diversity, and its ability to adapt differs among diverse varieties. Thus, clarifying the physiological status of N, P, and K is necessary not only for nutrient management but also to improve the breeding research of *C. oleifera* in the future.

The nutrient status assessment based on the chlorophyll content or diagnosis and recommendation integrated system (DRIS) in *C. oleifera* has been reported (Gao *et al*., 2016). However, it is unsatisfactory to begin illuminating the nutrient characteristics of diverse genetic resources without a full consideration of the interactions among the elements. The ionome is a dynamic network of elements that is controlled by the physiology and biochemistry of the plant (Salt *et al*., 2008). It can be used to assess the nutrient status of the plant based on the synergism and antagonism between the elements (Williams & Salt, 2009; Parent *et al*., 2013). A multivariable ionomic signature for the Fe response status of *Arabidopsis* has been previously established (Baxter *et al*., 2008). Additionally, it has been reported that the phylogenetic variation of barley, cucumber, and tomato grown in Fe deficient soils can be distinguished by the shoot ionome (Pii *et al*., 2015). So far, the effect of the N, P, and K deficiency on mineral elements has also been studied (Cao *et al*., 2016). However, few attempts have been made regarding the physiological status assessment and prediction based on the ionome in *C. oleifera*.

Selecting the proper tissue for the physiological status assessment is quite indispensable. The ideal tissue for most ionome analysis can be derived from either the leaves or the seeds (Baxter, 2010). For *Arabidopsis*, the young leaves of 5-week-old vegetative stage plants are usually used. Whereas for maize, on the other hand, the kernels can be utilized due to their uniform properties (Baxter *et al*., 2014). Due to the nonuniformity of the seeds, and variations in the parameters such as maturity, and oil and mineral elements content (Zhou *et al*., 2013), the leaves are usually taken as the ideal tissue for nutrient assessment in *C. oleifera* (Gao *et al*., 2016). However, appropriate sampling time points for the nutrient status assessment and prediction also need to be developed.

As a non-supervised dimension reduction method, Principal Component Analysis (PCA) attempts to select a small number of orthogonal coordinates to maximize the overall explained variation in the data. It is usually used for multi-dimensional data processing in ionome analysis. Additionally, it could solve the multicollinearity problems during the multivariate logistic regression. Using PCA coupled with multivariate logistic regression, the Fe status in *Arabidopsis* was successfully predicted (Baxter *et al*., 2008).

The present study aimed to explore a statistical method to predict the N, P, and K status of *C. oleifera* using the leaf ionome. A total of 11 elements in the leaves of the sand-cultured seedlings under N, P, or K deficiency were investigated using ICP-MS. The principal component variables of the leaf ionome were analyzed in response to the N, P, or K deficiency based on the altered ions with simultaneous changes in the concentrations of N, P, and K. Subsequently, the classification models for the *C. oleifera* seedlings responding to the N, P, and K supply based on the logistic regression were developed. Thus, the study provides a new path for nutrient research and the screening of germplasm resources of *C. oleifera* using ionome analysis in the future.

Materials and Methods

Plant materials and experimental design: Two-year-old cutting seedlings of *C. oleifera* 'Changlin 4' were selected for this study. The well-grown seedlings over 18 cm in height were transplanted into trays (10.3 ×7.0 cm) with 21 holes each for sand culture at 25 days after sprouting, in April 2017. The roots were fully cleaned with deionized water before transplantation. For all experiments, a total of 48 trays were prepared. The plants were bottom watered with Hoagland's solution (5 mM KNO_3, 5 mM $Ca(NO_3)_2.4H_2O$, 2 mM $MgSO_4.7H_2O$, 1 mM KH_2PO_4, 46 μM H_3BO_3, 9 μM $MnCl_2.4H_2O$, 0.8 μM $ZnSO_4.7H_2O$, 0.3 μM $CuSO_4.5H_2O$, 0.12 μM $Na_2MoO_4.2H_2O$, and 40 μM FeNaEDTA). After three weeks, a total of 40 trays were randomly selected for the nutrient deficiency experiments. The other eight trays were taken as the control. The temperature in the greenhouse was controlled at 25°C and the moisture was above 70%.

In the N deficiency experiment, the N level of Hoagland's solution was controlled at 0.5 mM. However, 5 mM $Ca(NO_3)_2{\cdot}4H_2O$ were replaced with 0.5 mM KNO_3, 4.5 mM KCl and 5 mM $CaCl_2$. In the P deficiency experiment, the P level of Hoagland's solution was controlled at 0.01 mM. Here, 1 mM KH_2PO_4 was replaced with 0.01 mM KH_2PO_4 and 0.99 mM KCl. In the K deficiency experiment, the K level of Hoagland's solution was controlled at 1 mM while 5 mM KNO_3 was replaced with 5 mM $NaNO_3$. In the Mg and Mn deficiency experiment, the Mg and Mn were provided at 0 mM. Additionally, $MgSO4{\cdot}7H_2O$ and $MnCl_2{\cdot}4H_2O$ were removed.

All the plants were watered once every two days with nutrient solution (1 L/tray). The pots were rotated horizontally to reduce the gradient effects of light, temperature, and humidity.

Sampling and measurement: Leaves from spring shoots were sampled for ICP-MS analysis after two weeks of stress treatment when the spring shoots were fully developed and the nutrient composition was relatively stable at 60 days after sprouting (Fig. 1). The sampled fresh leaves were washed using 18 MΩ water before drying at 60°C for 96 h. The dried samples (0.5 g) were digested using concentrated HNO_3 (ultrapure grade, 69 %, Merck, Darmstadt, Germany) at 112°C for 5 h. The total P, K, Ca, Mg, Fe, Mn, B, Zn, Cu, and Mo ion concentrations were determined using ICP-MS (Nexion 300D, Perkin Elmer, Shelton, CT, USA) as described by Watanabe *et al.*, (2016). Additionally, the N content was determined using the Kjeldahl method. All the determinations were completed at the Quality Testing Center for Non-wood Forest Products of State Forestry Administration (Hangzhou, China).

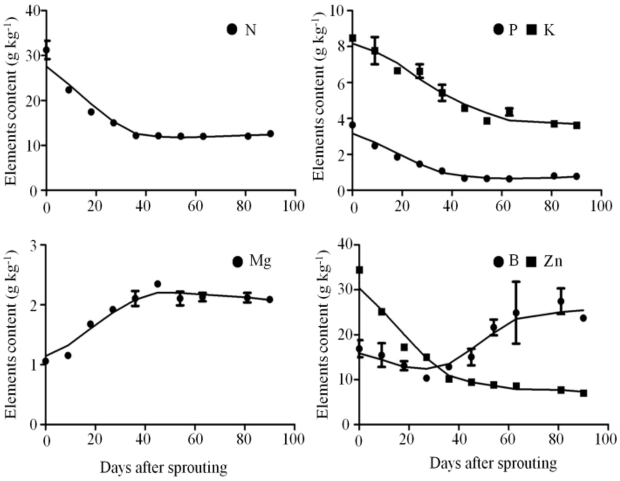

Fig. 1. Changes of mineral elements N, P, K, Mg, B and Zn in *C. oleifera* leaves on spring shoot. The values are the means ± SE of three replicates, and each replicate consisted of a pool of five plants.

Statistical analysis

Statistical differences in the ion concentrations were assessed by variance (ANOVA) and a significance level of $p<0.05$ was considered. The PCA and logistic regression analysis were used to predict the different physiological status. All statistical calculations and graphs were performed using IBM SPSS 19.0 and GraphPad Prism 5 (USA, GraphPad Software, Inc.).

Results

The ionomic variation observed under N, P, or K deficiency: The interactions between the elements caused by synergism and antagonism have been demonstrated. On the other hand, the correlations between the elements within a tissue appear to be highly variable between species, tissues, and environments (Baxter *et al.*, 2012). The ionomic variation in response to N, P, and K needs to be confirmed for the physiological status assessment using the leaf ionome in *C. oleifera*. The N, P, K, Ca, Mg, Fe, Mn, B, Zn, Cu, and Mo ion concentrations in the leaves from 40 randomly selected seedlings in each of the N, P, and K deficiency experiment were determined. Significant relationships among the ions were found. Not only were the leaf N, P, and K ion concentrations decreased, but the B, Cu, Fe, Mg ion concentrations were also significantly altered under the N, P, and K deficiency (Fig. 2). The K and B ion concentrations were decreased while the Cu was significantly increased with the N decline in the N deficiency treatment. The concentrations of Fe and Cu increased while that of N was decreased significantly with the P decline in the P deficiency treatment. Additionally, the concentrations of Fe and Cu were increased while those of N, B, and Mg were decreased significantly with the K decline in the K deficiency treatment.

Multicollinearity diagnostics and PCA analysis: Here, the coordinated variational ions were used to derive the classification models based on logistic regression (Pii *et al*., 2015). Additionally, the data of the coordinated variational ions were collected from 200 randomly selected seedlings, 100 seedlings for the control and 100 seedlings for nutrient deficiency. Due to the interactions between the ions, a severe multicollinearity problem was observed when the ions were taken as independent variables during the logistic regression. The condition index and the eigenvalue were far beyond the tolerance for multivariable logistic regression in the three tested groups of N, P, and K (Table 1). This made the derived prediction model unstable and inaccurate (Johnson & Wichern, 2008). PCA was introduced to solve the multicollinearity problem and reduce the dimensions of the multivariables based on the leaf ions, such as the N, K, B, and Cu ions in the N deficiency experiment, the P, Fe, Cu, and N ions in the P deficiency experiment, and the N, K, Fe, Cu, B, and Mg ions in the K deficiency experiment. Three principal components were extracted for each group by the PCA and the accumulative contribution was up to 91.138%, 88.971%, and 64.519%, respectively. Additionally, the extracted principal components provided a reasonable separation between the control and the nutrient deficiency seedlings (Fig. 3).

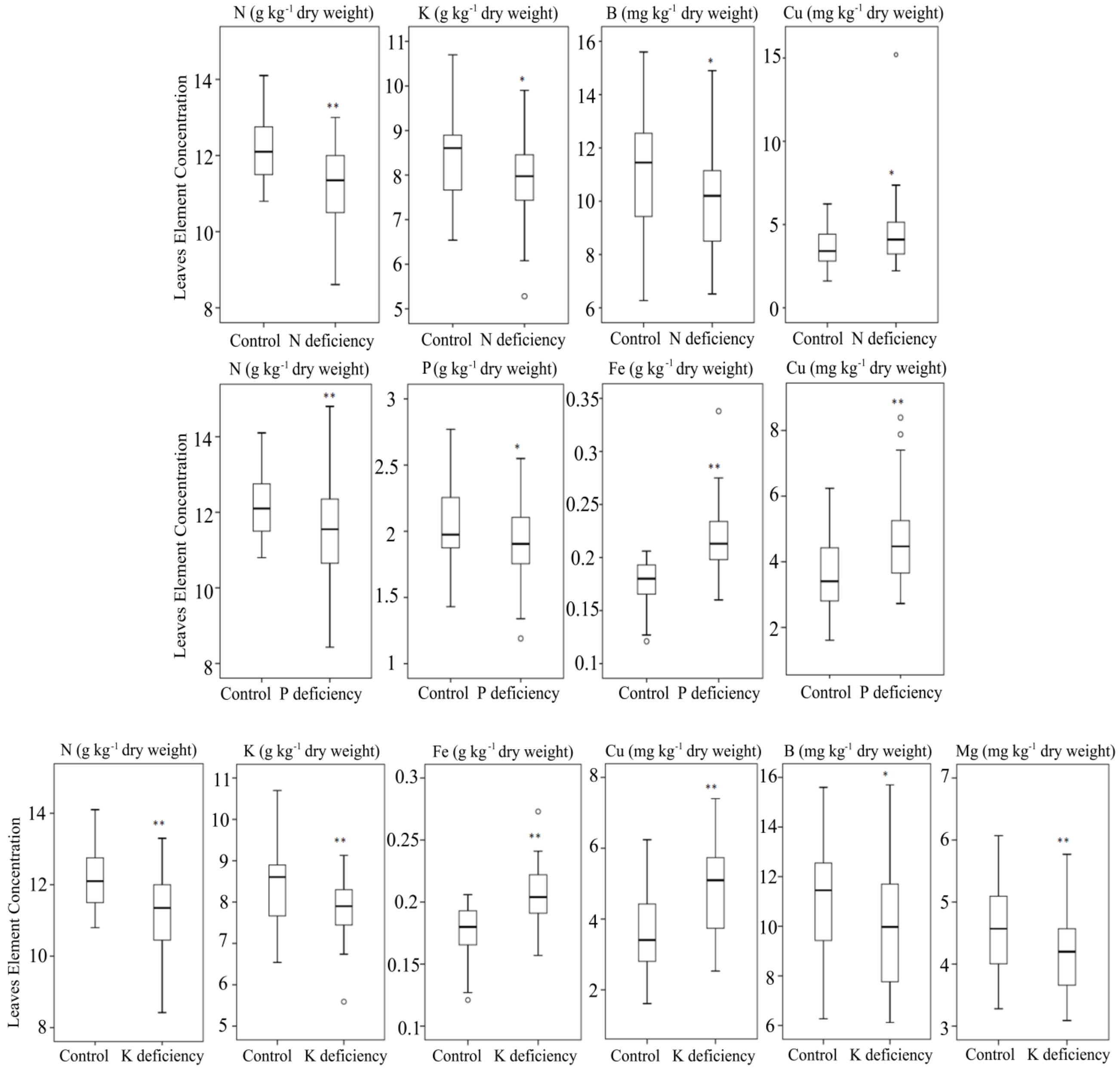

Fig. 2. Effects of N, P, K deficiency on leaves ionome of *C. oleifera* seedlings. The box represents the interquartile range, the bisecting line represents the median and the dots represent outlier points. Significant difference between Control and nutrient deficiency treatment was determined by T-test and marked as *(p<0.05) and ** (p<0.01). The seedlings (n =40) were subjected to 0.5 mM NO_3^-, 0.01 mM $H_2PO_4^-$ and 1 mM K^+ for 2 weeks, respectively.

Table 1. Multivariable collinearity diagnostics based on N, P, K, B, Cu, Fe and Mg concentrations.

Dimension	N group		P group		K group	
	Eigenvalue	Condition index	Eigenvalue	Condition index	Eigenvalue	Condition index
1	3.831	1.000	3.900	1.000	4.665	1.000
2	0.144	5.166	0.063	7.856	0.957	2.208
3	0.020	13.965	0.028	11.775	0.264	4.202
4	0.006	26.116	0.008	21.542	0.083	7.514
5	—	—	—	—	0.023	14.370
6	—	—	—	—	0.008	23.965

Dependent variable: N

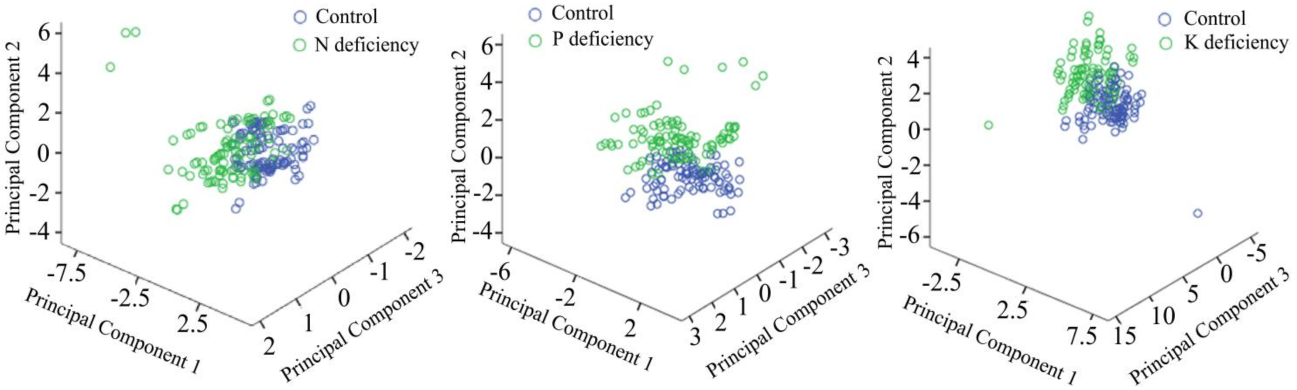

Fig. 3. Leaves ions analysis of N, K, B, Cu under N deficiency, N, P, Fe, Cu under P deficiency, N, K, Fe, Cu, B, Mg under K deficiency based on PCA. The analysis was performed on data from *n* =100 plants for each treatment.

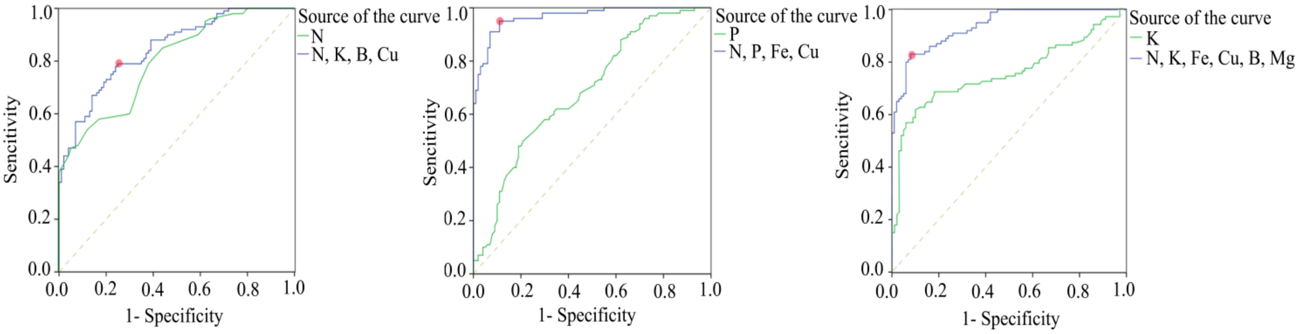

Fig. 4. Receiver-operator curves of the various logistic regression models. Green line, model built by using solely leaf ion. Blue line, model built by using leaf ionome. Dotted reference line, a model with no predictive ability. Red dot means the best cutoff level during prediction.

Multivariable logistic regression and receiver-operator curves: In the test, data from 200 randomly selected seedlings were evaluated, as mentioned above. The ion concentrations of the leaves contained information that could discriminate the seedlings based on their N, P, or K status by PCA. The classification models which could classify the seedlings based on their response to the N, P, or K deficiency were derived by taking the extracted principal components as the predictor variables during the logistic regression. The regression equations in the test were as follows. $logit(P)_N = 0.169 - 0.942PC_1 + 0.568PC_2 + 2.031PC_3$, $logit(P)_P = 0.437 - 1.289PC_1 + 4.215PC_2 - 1.593PC_3$, and $logit(P)_K = 0.146 - 1.781PC_1 + 1.911PC_2 - 3.339PC_3$. A logistic regression takes the values of the principal components containing leaves with coordinated variational ion information as the input predictor variables. Subsequently, the probability of showing a response to nutrition deficiency is given as the output. In addition, the probability was calculated by $EXP(logit(P))/(1 + logit(P))$.

Next, receiver-operator curves were used to check the predictive ability of the built models. To perform the best classification of the different N, P, and K status, the cutoff level was determined by the largest difference between Sensitivity and 1 - Specificity was dotted red on the curves (Fig. 4). The AUC values obtained in the logistic regression models built using the leaf ionome and the models built using solely ion were compared. The model with the higher AUC values was determined to have the better predictive ability. For the N status prediction, there was no statistical significance ($p>0.05$) in AUC values between the logistic regression model built using leaf N, K, B, and Cu and the model built using solely N. For the P and K status prediction, the AUC values of the models built using the leaf ionome were significantly higher ($p<0.01$) than that of models built using the ion alone. Additionally, the models built using leaf ionome produced a >85% accuracy in discriminating the seedlings under the P and K deficiency. On the other hand, the models built using a single ion only produced a <61% accuracy.

Table 2. Prediction of P status of *C. oleifera* seedlings grown in altered nutrient solutions using the models built by using leaf ions and solely P.

Element (Hoagland's)	Concentration	Plants predicted to be P-deficiency	
		Model built by using N, P, Fe and Cu	Model built by using P
NO_3^- (5 mM)	0.5 mM	6	18
K^+ (5 mM)	1 mM	4	22
Mg^{2+} (2 mM)	0 mM	8	20
Mn^{2+} (9 μM)	0 mM	4	25

40 plants were randomly selected for each treatment

Table 3. Pearson's correlation matrix of the elements concentration in leaves of *C. oleifera* seedlings fertilized with Hoagland's nutrient solution by sand culture.

	N	P	K	Ca	Mg	Fe	Mn	B	Cu	Zn	Mo
N	1	0.492^{*}	0.501^{**}	-0.009	0.230	0.435^{*}	-0.291	0.560^{**}	0.214	0.341	0.299
P		1	0.310	-0.074	0.169	0.492^{*}	-0.281	0.374^{*}	0.125	0.319	0.167
K			1	0.325	0.514^{**}	0.539^{**}	-0.043	0.780^{**}	0.238	0.421^{*}	0.332
Ca				1	0.625^{**}	0.560^{**}	0.459^{*}	0.442^{*}	-0.102	0.059	-0.086
Mg					1	0.594^{**}	-0.041	0.609^{**}	-0.213	0.345^{*}	0.027
Fe						1	0.017	0.661^{**}	0.132	0.337	0.289
Mn							1	-0.138	0.171	-0.301	-0.305
B								1	0.151	0.454^{**}	0.417^{*}
Cu									1	-0.017	0.403^{*}
Zn										1	0.238
Mo											1

Data from n = 55 plants. Significant difference was marked by *(p<0.05) and **(p<0.01)

Test of the logistic regression models built using the leaf ionome: To test whether the logistic regression model built using the leaf ionome was specific or not, the model for P status prediction was chosen for further evaluation. A total of 160 seedlings under N, K, Mg, or Mn deficiency and 40 seedlings for each treatment were used in the test. The P physiological status of the 160 seedlings was assessed using the derived P status prediction model. It was revealed that the accuracy was over 80% using the P status prediction model built by the leaf ionome. On the other hand, no prediction power was observed using the model built using the sole P ion (Table 2).

Discussion

Interaction among ions: A balanced supply of inorganic elements is needed for optimal plant growth and development. Also, the mechanism underlying the homeostatic control of ions in an organism is strongly interrelated (Eide *et al*., 2005). The interaction among the ions, important for plants to adapt to specific conditions, has been proven in many plant species (Webster *et al*., 2009; Quadir *et al*., 2011; Baxter *et al*., 2012; Wu *et al*., 2013). For example, it has been reported that the deficiency of N leads to the coordinated variation of NO_3^-, Cl^-, and PO_4^{3-} (Loudet *et al*., 2003). P deficiency alters the concentrations of the B, P, Cu, and Zn ions (Baxter *et al*., 2008). K promotes the absorption of NO_3^- and is significantly related to the B and Cu ions (Pii *et al*., 2015). Additionally, these correlations among ions seem to be highly variable between species (Quadir *et al*., 2011; Baxter *et al*., 2012). Correlations among the ions in the leaves of *C. oleifera*, a typical aluminum (Al) and Mn accumulator that is grown in acidic soil (Zhuang, 2008; Zeng *et al*., 2011), need to be observed. Our research showed that the N, P, K, and B ions exhibited significant positive correlation with each other in the leaves of *C. oleifera*. Besides, in addition, the K ion showed significant positive correlations with the Mg and Zn ions (Table 3). However, nutrient stresses like N, P, or K deficiency tend to break this balance among ions and cause alterations in N, P, K, B, Fe, Cu, and Mg ion concentrations in the leaf (Fig. 2). Like Webster *et al*., (2009), who reported that correlations among ions such as P, Fe, and Zn usually depended on the ion concentrations, some of the correlations among the ions were also altered under nutrient stress in the present study. For example, it was observed that the leaf Cu concentrations showed a significant increase under the N, P, and K deficiency, the leaf Zn concentrations remained steady under K deficiency, and the Fe ion showed a negative correlation with the P, K, and Zn ion concentrations. It has been proved that some antagonism exists between the P, Zn, and Fe ions. Also, the Zn ion could be transported by the iron-regulated transporter 1 gene, *IRT1* (Vert *et al*., 2002), while the molecular basis for the interactions between the N, P, and K nutrition and the B, Fe, Cu, and Mg ions is less clear.

The use of leaf ionome: In addition to the plant nutrition status prediction, the alterations and correlations between the ions in the ionome could also be used for the screening of mutants and gene identification. For example, 51 mutants of *Arabidopsis* were discovered using ionome analysis based on 18 elements.

Additionally, the genes encoding the Fe-citrate transporter and ferric chelate reductase in *Arabidopsis* were identified using ionome analysis (Lahner *et al*., 2003; Pii *et al*., 2015). The yield of *C. oleifera* is severely limited because of the widespread shortage of N, P, and K in the red soil of southern China. In recent times, an exploration of high nutrient-efficient resources and scientific nutrient management has gained importance with reference to *C. oleifera*. The N, P, and K physiological status assessment based on the ionome information could provide a new path for nutrient research and the screening of the germplasm resources of *C. oleifera*.

Prediction ability of the built logistic regression models: The prediction ability of the built logistic regression models responding to N, P, and K was different in the present study. The P and K status prediction models built using the leaf ionome performed far better than the models built with the use of single ion. However, no significant difference was found between the model built using the leaf ionome and the model built using a single ion for the prediction of N status (Fig. 4). Thus, it was revealed that the leaf N ion concentration showed greater sensitivity to nutrient (N) deficiency than P and K in the variety ‘Changlin 4’ of *C. oleifera*. According to our previous study, the nutrient absorption and use efficiency were quite different among the varieties of *C. oleifera*. Therefore, it was inferred that the difference of the leaf ions responding to the specific nutrient deficiency could be attributed to the genetic differences among the diverse varieties.

Conclusions

The models to predict the N, P, and K status of *C. oleifera* using the leaf ionome based on the PCA and the logistic regression were developed. Additionally, these models could classify the seedlings based on their response to the N, P, and K deficiency. Except for the N prediction model, the P and K status prediction models using the leaf ionome performed far better than the models using sole ions. These produced a >85% accuracy in discriminating the seedlings under nutrient deficiency. In addition, the P prediction model exhibited excellent specificity in the tests under N, K, Mg, or Mn deficiency.

Acknowledgements

This study was funded by the National Natural Science Foundation of China “*Evaluation of oil-tea Camellia plant’s nitrogen, phosphorus, potassium status based on leaf ionome* (Grant Number 31600551)”.

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(Received for publication 5 April 2018)