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Volume 3 | Issue 1 | January 2021 | Page1-51

Journal of Botanical Research

Contents

ARTICLE

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ARTICLE

Interspecific Variation and Phylogenic Architecture of *Pinus densata* and the Hybrid of *Pinus tabuliformis*×*Pinus Yunnanensis* in the *Pinus densata* Habitat: an Electrical Impedance Spectra Perspective

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ABSTRACT

We evaluated a novel and non-destructive method of the electrical impedance spectroscopy (EIS) to elucidate the genetic and evolutionary relationship of homoploid hybrid conifer of *Pinus densata* (*P.d)* and its parental species *Pinus tabuliformis (P.t)* and *Pinus yunnanensis (P.y)*, as well as the artificial hybrids of the *P.t* and *P.y*. Field common garden tests of96 trees sampled from 760 seedlings and 480 EIS records of 1,440 needles assessed the interspecific variation of the *P.d, P.t, P.y* and the artificial hybrids. We found that (1) EIS at different frequencies diverged significantly among germplasms; *P.y* was the highest, *P.t* was the lowest, and their artificial hybrids were within the range of *P.t* and *P.y*; (2) maternal species effect of EIS magnitudes in the hybrids and *P.d* was stronger than the paternal species characteristics; (3)EIS of the artificial hybrid confirmed the mid-parent and partial maternal species characteristics;(4) unified exponential model of EIS for the interspecific and hybrids can be constructed as $|Z|=Af^{-B}$; (5) cluster analysis for species and hybrid combinations in total corroborated with the previous hybrid model of *Pinus densata.* Our non-destructive EIS method complemented the previous finding that *Pinus densata* was originated from *P.t* and *P.y*. We conclude that the impedance would be a viable indicator to investigate the interspecific genetic variations of conifers.

1. Introduction

Pinus densata(P.d) is a natural homoploid hybrid conifer originated from two ancestry species (i.e., *Pinus tabuliformis* and *Pinus yunnanensis*), as evidenced by previous genetic studies of isozyme, cpDNA, and mtDNA [1]. The natural habitat of *P.d* ecologically diverged from its parental species *Pinus tabuliformis* or *Pinus yunnanensis*. It has the highest habitat range among the Asian *Pinus* species and its habitats ranges from 2,800 to 4,200 above sea level

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(a.s.l.) on the Tibet Plateau, China; *Pinus tabuliformis* distributes in the mountainous, warm and temperate forests in northern China at a lower altitude below 2,200m a.s.l.; the habitat of *Pinus yunnanensis* locates in the southwest of the *Pinus tabuliformis* 'range, from 1,000m to 2,800m a.s.l.[1-4].

Previous phenotypic studies of the interspecific adaptation and eco-physiology of the three pine species indicate greater fitness and survival rates of *Pinus densata* in stress tolerance such as to lower temperatures [5], and drought, in the light of greater dry mass production and long-term water-use-efficiency compared to the parental conifers [2]. Gao, Gao (6) found that *Pinus densata* had a stronger antioxidant process under drought than the parental species, while *Pinusyunnanensis* was drought sensitive.

In ecological genetic studies, Mao and Wang (1) found *Pinus densata* more adapt to high- altitudinal habitat in terms of the niche characteristics, such as temperature seasonality, growing-degree days. Mao, Li (7) studied eight morphometric traits of cone and seeds in *Pinus densata* and the parental species and concluded that *Pinus densata* is more reproductively successful in the natural habitat than the local *Pinus tabuliformis* and *Pinus yunnanensis*. Among the three *Pinus* species, the cross barrier is weak and the fitness differences are determined by local adaptation [8]. These phenotyping assessments are laborious to compare the interspecific variation, involving field sampling, common garden trials, experimental measurement and destructive evaluation methods.

Biological electrical impedance spectra (EIS) provides an alternative method compared to destructive procedures to monitor physical changes of tissues in trees such as *Pseudotsuga menziesii* [9] and *Picea glauca* [10]. Recently, impedance measurements have been frequently applied in root studies including surface area and mycorrhizae [11-15]. Repo, Laukkanen (16) studied impedance under 40Hz to 340 kHz to assess tree root growth. Then, Repo, Korhonen (13) studied root colonization of *Pinus sylvestris* L. seedlings with mycorrhiza fungi (*Hebeloma sp*. and *Suillus luteus*) under 5Hz-100kHz; and they found 13% to 27% correlated change for the real and imaginary parts under cold vs high-temperature treatment; under mycorrhiza treatment, there are 30%-39% correlation change in the real part, and 28%-38% in the imaginary part of impedance, respectively. Later, Repo, Korhonen (17) used similar 5-100kHz frequency of the voltage to measure the impedance of *Pinus sylvestris* L. under hardiness treatment on roots and developed a new method to categorize root frost injuries by EIS.

Needles are the photosynthetic organs that are easier to collect and measure than other organs such as roots. The structure and electrical-physiological signatures of needles vary among germplasms or species [18,19]. EIS is a potential technique to detect such a signature of needles, among germplasms or species, although previous application was frequently in the stem and root studies [11-13].Zhang, Li (20) employed EIS to assess the shoot and needles of *Pinus Bugeana* and reported greater correlations with the electrolyte leakage under frost hardiness (r = -0.8~0.9).

EIS is also a genotyping tool for crop plants. Kocheva, Georgiev (21) measured leaf impedance under seven Hz to two kHz to assess varietal difference and found low impedance genotypes with higher ion leakage in *cultivar Prelom* variety. In genetic mapping, EIS can build the phenotypic association with the genotypes of quantitative trait locus with statistical models [22].

In this study, we explored the feasibility of the non-destructive EIS method for assessing the genetic variation among relative species by capturing the needles signature potentially due to evolution and local adaptation. This paper reported the variation and trend of EIS parameters by testing the needles of different germplasms among artificial hybrids of *Pinus* tabuliformis(P.t), and *Pinus yunnanensis* (P.y), as well as the homoploid hybrid,*Pinus densata* (P.d).The basic hypothesis is that EIS signatures of the low or high frequency could reflect the genetic variations between the homoploid hybrid conifer (*P.y*),and related parental species. Our objectives are(i) to explore the interspecific variation of EIS among *P.d, P.t, P.y* and the artificial hybrids; (ii) to assess the association between genetic variation and EIS parameters within the hybrid families; (iii) to test the parental effects on the artificial hybrids in terms of the EIS variability. By examined the feasibility of EIS to study the systematic evolution and the adaptation of homoploid hybrid conifers, our study provided a novel technique and strategy for forest genetics and adaptation research.

2. Materials and Methods

2.1 Plant Materials and Study Site

2.1.1 Artificial Interspecific Hybrids, *Pinus tabuliformis* and *Pinus yunnanensis*

We sampled six clones of *Pinus tabuliformis* as the female parents, five *Pinus yunnanensis* as the pollen donors from Kunming, Yunnan, China to conduct the controlled pollination in the seed orchard at Ningcheng, Heilihe, Chifeng City, Inner Mongolia, China. To construct research pedigree, we employed a test cross mating design to obtain 30 hybrid families, within which 13 hybrid families were subjected to the common garden experiment

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based upon the seed quality.These 13 families were coded as from 401, 402, to 413 in the experiment and analyses. We focused on both the inter- specific variations and among-family variations of the hybrids.

The *Pinus tabuliformis* germplasms were open-pollinated half-sib families developed from the six female parents that produced the artificial hybrids. The *Pinus yunnanensis* population was developed from the five pollen donors that formed five half-sib families and the donors were used for producing the artificial hybrids. There were 30 *Pinus densata* trees sampled in the stands in Linzhi City, Tibet, China. In the sampled stands, *Pinus densata* is naturallydistributed without other conifers stands in the same genus.

2.1.2 Common Garden Field Trial

We constructed common garden experiments in the native range of *P.d* to compare different species and sampled seedlings within the trial for EIS assessment. The common garden field trial locates at the research nursery of the College of Resources and Environment, Tibet College of Agriculture and Animal husbandry, at Bayi County, Linzhi City, Tibet, China (93°25' E, 29°50W, 2,900m a.s.l.). The study area has a semi-humid monsoon highland climate(mean annual temperature8.5 ℃ ; min annual temperature -15.3 ℃ ;max annual temperature 30.2 ℃ ; annual precipitation 654.1mm, from April to October; annual sunlight hour 2,022hours; annual frost-free period >180days). The nursery was built on the former flood bed. The soil was sandy loam with a pH ranging from six to seven and a depth more than 60cm. The fertility of the soil was from medium to low. The native *Pinus densata* grows normally in the nursery area.

2.1.3 Seedling Preparation and Experimental Design

The common garden field trial was established on a high bed of 5m length from east to west, 1.1m width and10cm height. A randomized complete block design (RCBD) was employed at the trial. There are 4 blocks, 13 artificial hybrid families,and three pure species as control (*Pinus densata*, *Pinus* tabuliformis, and *Pinus yunnanensis*) with two seedlings within each of the three pure species; for each treatment (13*1 hybrid families+ 3*2 families), there are ten seedlings planted per treatment level for the artificial hybrids and ten seedlings for treatment of control species within each block. The row distances and maintenance conditions were consistent for each block. Thus, the complete experiment contained 19families (i.e., 13+3 treatment levels) x 10 trees per plot x 4 replication, 760 seedlings in total. The common garden experiment and progeny structures were reported in previous studies [23], but a new set of individual trees were selected from the field trial for this study.

2.2 Impedance Measurement of Needles

Impedance measurement was conducted on the secondary growth needles of age-2 of the seedlings. The sampling process of test trees was as following, from three randomly selected blocks, choose (13+3) treatments (germplasms) in total, and select six trees per treatment. Impedance measurement was carried out for five times per seedling, and in each time, three normally-growing needles were picked. For each germplasms, 90 needles were measured. The total measurements were as 16 germplasms x 6 trees x 5times x 3 needles = 1,440 needles.

2.3 EIS Measurement Procedures and the Frequency Responses

We connected the impedance analyzer (TH2828S, Changzhou Tonghui Electrical Co. Ltd., Jiangsu, China) with two stainless steel electrodes (the red and black), and calibrated the open and short circuits of the impedance analyzer. Then we sampled two healthy needles from *Pinus tabuliformis*, *Pinus yunnanensis*, *Pinus densata*, and the artificial hybrids at the branches that were at one-third of the tree height aboveground (i.e., ~1mm in diameter and ~1 cm in length, no chlorosis or pathogenic syndromes). The voltage electrodes were clippers and were connected directly to the needles with about one centimeter between each clipper without additional conductive media. The voltage was set to ten mV and the impedance was measured at 53 frequencies between 1Hz to 100MHz to draw the impedance curve. We plotted the EIS trend from one to 80Hz as well as from one kHz to 100kHz. The EIS from one Hz to 80Hz showed more variability comparing to the range from 80Hz to 100kHz and the trajectories were similar in the two spectrum ranges. To address the environmental effects such as the light and temperature, we repeated the measurement for five times at the same location of branches from the morning to the afternoon. We compared the potential polarization effect under low frequency measurement with the higher frequency measurement (Supplementary materials).

We aimed to test the feasibility of impedance parameters for testing species genetic variations. Here we assumed that the physiological responses were negligible and consistent when needles were connected to the current, e.g., the physiological change when the needle cells were ruptured. The frequencies for impedance mea-

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surement were between one Hz and 100kHz. We showed both the lower (1-80Hz) and higher frequency results (1k -100kHz). The original status of the needles was consistent during the measurement without cutting the needles off the trees. Nyquist plots of the impedance for different germplasms were constructed to present the interspecific variations. Both the real (Z', resistance) and imagery part (Z", reactance) of impedance were compared in the Nyquistplot among species.

2.4 Statistical Analyses

We took the average impedance of three needles for the interspecific analyses of variance (ANOVA), followed by Tukey's test for multiple comparisons with R software [24]. For interspecific variation, we used the following linear model:

$$y_{ijk} = \mu + S_i + f_{j(i)} + e_{ijk} \quad (1)$$

where y_{ijk} is the sampled impedance reading of individual tree; μ is the grand mean; S_i is the i-th species, which includes *P.t, P.d, P.y* and the artificial hybrids; $f_{j(i)}$ is the j-th family nested in i-th species; e_{ijk} is the random residual of the k-th tree of ji-th family. For comparing the hybrid families, we applied another linear model as following:

$$y_{jk} = \mu + I_j + e_{jk} \quad (2)$$

where y_{jk} is the sampled impedance reading of each family; μ is the grand mean; $I_j$is the j-th hybrid family, which includes *P.t, P.d, P.y*, and the artificial hybrids; e_{jk} is the residual of the EIS average of k-th tree in j-th hybrid family.

We also constructed the unified exponential model of EIS is as following:

$$|Z| = Af^{-B} \quad (3)$$

where A and B are the regression coefficients of species; A is also the amplitude (A>0); B is the exponential coefficient (0<B<1); |Z| is the impedance magnitude (kOhm); f is the frequency (Hz). The exact specific or phenotypic trait factors to modify A and B are still under studies.

To evaluate the EIS of interspecific kinships, the cluster analyses were conducted based on arithmetic means of EIS within six germplasms of three pine species and family means of each hybrid family. The coefficients of variation were calculated as the standard deviation divided by means of the germplasm- family group. Correlation coefficients 0.6 were the threshold value for hierarchical clustering. Ward's method was used to determine the Euclidean distances of impedance among different germplasms, which is the distance of all clusters to the grand average of the sample. The distances of impedance and branches of the dendrogram indicated the among-family and interspecific genetic structure inferred from the EIS. To depict the unified function between EIS and voltage frequencies we used non-linear regression with exponential independent variables (i.e., frequency). We used R software for the statistical analyses of variance components, regression and cluster analyses [24].

3. Results and Discussion

3.1 Among-family Impedance Variation and Impedance of Artificial Hybrids

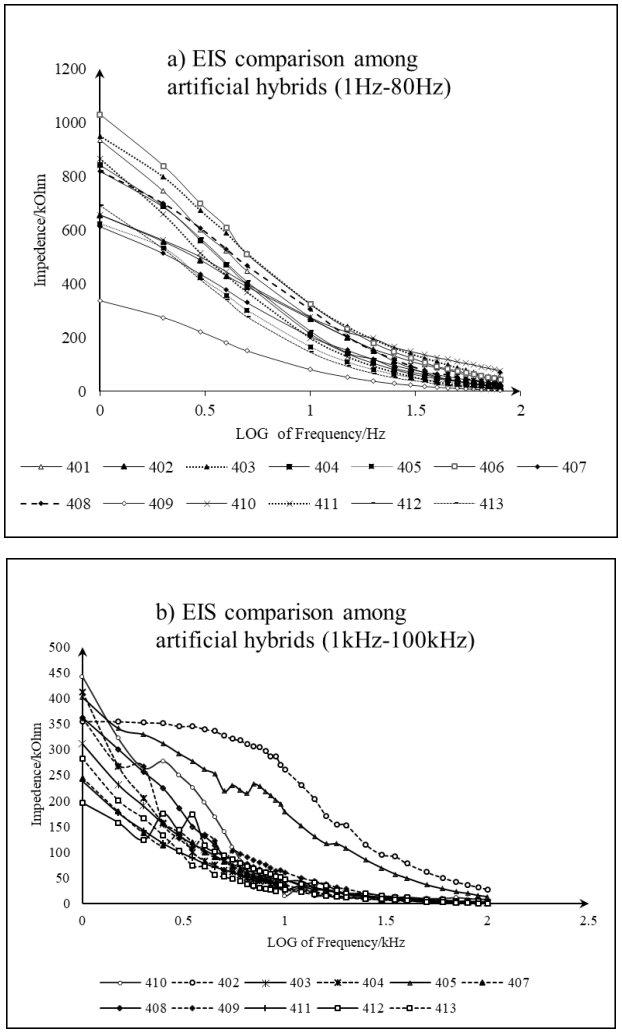

Figure 1. Impedance spectroscopy comparison for artificial hybrids

Note:
The frequency in a) is transformed by logarithm to base 10 ranging from zero to 80 Hz; the frequency in b) ranges from one to 100kHz.

The artificial hybrids of *Pinus tabuliformis* and *Pinus yunnanensis* showed a decreasing impedance when the

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frequencies of the external alternating electric field rise (Figure 1). The impedance values of all hybrids peaked at one Hz. When the frequency increased, the dropping speed of each hybrid varied. The gradient of impedance reached its maximum (400-1,500 kOhm) when the frequency changed from one Hz to five Hz. At 10Hz of the field frequency, the impedance curve inflected; and the impedance was only 30% of the initial value at one Hz. Then the declining speed of the impedance curve reduced and reached to 10% of the initial value at 50Hz.

We found a discernable genotypic variation of impedance between hybrid individuals when the voltage reached 30Hz. And the differences of impedance between hybrids kept stable when the frequency was greater than 30Hz. When the frequency was lower than 30Hz, the interspecific variation dropped, in which Hybrids 409 and 406 expressed of the lowest and the highest impedances, respectively. The interspecific variation was negligible as the frequency was lower than five Hz. To magnify the interspecific variation in the low frequencies, we took the log-transform for frequency (Figure 1). Figure 2 and Figure 3 showed impedance variation among hybrids and species in terms of the aggregated means of the bands of frequencies.

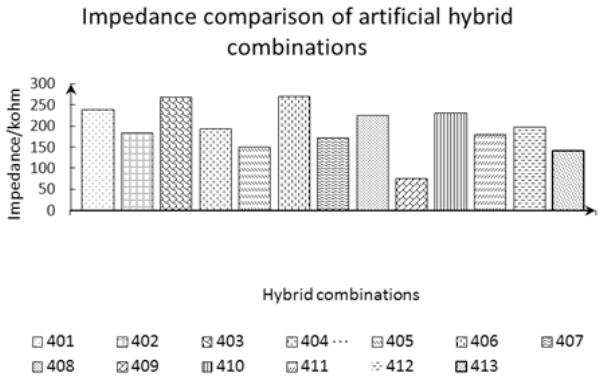

Figure 2. Impedance comparison of artificial hybrid combinations

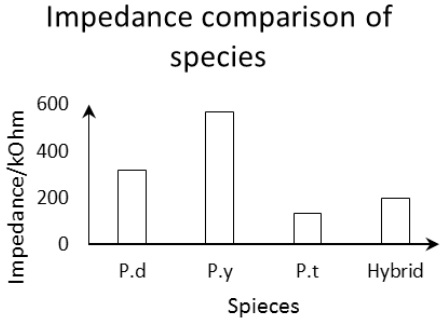

Figure 3. Impedance comparison of species (artificial hybrid)

3.2 Impedance Means and Variances of Hybrids

We did not find significant differences of impedance among hybrids in the analyses of variance (Table 1). The ratio of variance component among hybrids was 5.2%, while the ratio was 10.1% among individuals of each hybrid family. There was greater variation within the hybrid families than among the hybrids.

Table 1. Impedance variance analysis for artificial hybrids

		Impedance		
	DoF	Mean Square	Variance components/ %	Significance
Among the combinations	12	7875.9	5.2	ns
Among the individuals	39	4679.1	10.1	ns
Error	215	7133.8	84.7	

Notes:
*** P<0.001;*P<0.05; ns P>0.05.*

Table 2. Artificial hybrids mean and standard deviation of electrical impedance

ID	Impedance ± SD	Impedance similarity
401	84.13±98.98^{ab}	*P. t-like*
402	63.22±70.67^{ab}	*P. t-like*
403	78.00±44.24^{a}	*intermediate*
404	63.72±69.57^{ab}	*P. t-like*
405	83.67±74.27^{ab}	*intermediate*
406	95.98±94.39^{ab}	*P. t-like*
407	106.39±88.58^{a}	*intermediate*
408	77.30±95.45^{ab}	*P. t-like*
409	64.86±84.76^{b}	*P. t-like*
410	55.81±53.86^{ab}	*P. t-like*
411	92.57±72.56^{a}	*intermediate*
412	113.03±115.64^{a}	*intermediate*
413	54.21±55.50^{ab}	*P. t-like*

Note: P. t-like means the mean impedance of the hybrids is not significantly different from that P.t; Intermediate means the impedance value of the hybrid is significantly different but within the range of the parental species the P.t and P.y. The different letter signs "a" and "b" indicates significant differences (P<0.05) of average impedance between hybrids based Tukey test adjusted for multiple comparisons.

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We calculated the average impedance with three repeated measurements at a fixed frequency. The impedance of eight hybrids was similar as that of *Pinus tabuliformis* and five hybrids showed impedance between that of *Pinus tabuliformis* and *Pinus yunnanensis*; no artificial hybrids showed similar impedances as *Pinus yunnanensis*.

3.3 Interspecific Variation of EIS

Impedances of the hybrids was lower than *P.d*, although the Hybrid 404 at one Hz was higher than *P.d* (Figure 1). Among four hybrids, the impedance of *P.y* was the highest, followed by that of *P.d*. And the impedance of Hybrids was within the impedance range of the parental species (*P.y* and *P.t*), while the impedance of the Hybrids 405, 409, and 403 was lower than that of *P.t.* and *P.y*. The impedance curves became leveled off after 20 - 40 Hz (Figure 4). *P.d* and hybrids' curves were close to *P.t*. The variation among germplasms groups decreased with increasing frequency. However, the coefficient of variation among species groups increase by frequency and leveled off after 60Hz except for the EIS of hybrids (Figure 5).

Besides the impedance pattern of the frequency from one Hz to 80 Hz, from one kHz to 100 kHz, we tested the impedance of all germplasms. The curve trend and shape obtained were similar to the previous low-frequency EIS. We found the trajectory of impedance responses from one

Table 3. Impedancemean and variance comparison for artificial hybrid, parental species, and *Pinus densata*

Frequency	Mean±standard deviation				Variance components ratio/%	
	P.t	AH	*P.d*	*P.y*	Among species	Residual
1 Hz	690.8±265.4	754.1±324.8	1,067±388.8	1,450±418.8	32.2**	67.9
2	549.2±244.5	621.9±283.2	844.5±273.4	1,195±380.4	29.8**	70.2
3	434.8±218.7	517.6±253.5	695.3±218.2	1,017.8±344	29.1**	70.9
4	356.4±200.0	443.7±232.3	603.4±203.7	911.1±324.9	29.8**	70.2
5	300.2±185.4	383.3±213.5	532.9±197.7	835.9±314.5	31.3**	68.7
10	158.3±119.8	232.4±163.1	367.8±177.6	652.3±293.1	37.7**	62.3
15	103.3±87.3	163.8±133.4	293.4±165.6	562.4±269.3	42.6**	57.2
20	74.4±66.2	127.4±115.5	245.5±160.9	506.3±252.3	45.2**	54.8
25	51.2±52.7	101.0±97.5	213.5±156.2	466.0±238.7	48.9**	51.1
30	43.9±40.1	84.2±87.3	190.6±151.4	434.1±228.2	48.9**	51.1
35	35.8±32.7	71.9±80.1	171.9±148.4	407.3±219.0	51.0**	49.0
40	26.9±29.4	61.6±73.9	157.4±146.1	385.6±213.8	51.6**	48.4
45	24.6±22.8	55.1±70.6	147.9±143.0	365.5±207.9	48.3**	51.7
50	22.2±20.5	48.9±65.9	139.6±140.8	347.4±203.0	51.1**	48.9
55	19.4±18.6	43.7±62.1	132.1±137.3	331.5±197.5	51.2**	48.8
60	17.2±16.0	39.4±58.7	125.0±133.5	316.4±190.2	51.3**	48.7
65	15.7±14.0	35.4±56.2	119.1±131.4	304.1±184.0	51.5**	48.5
70	13.9±11.6	32.2±53.3	128.4±127.1	287.2±181.3	51.0**	49.0
75	12.2±9.8	30.6±50.9	123.1±125.7	276.7±177.8	50.55**	49.5
80	11.1±9.0	26.9±47.8	117.6±123.5	265.4±174.8	50.45**	49.6

Note:
**, P<0.05; **, P<0.01; P.d, Pinus densata; P.t,Pinus tabuliformis ; P.y, Pinus yunnanensis; AH, a hybrid of Pinus tabuliformis × Pinus yunnanensis.*

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Hz to 100Hz, and less variability occurred from 80Hz to one MHz. In the frequency ranging from one kHz to 100 kHz, the hybrids showed similar trajectories as that in the low frequency.

3.4 Variance Comparison of Impedance

The interspecific variations of impedance were significant at different frequencies (Figure 2). Impedance was an effective indicator to differentiate germplasms due to the physiological responses and activity under the electromagnetic fields. The variance component among species reached to 29% at one Hz to 30Hz; and the variance ratio between species exceeded 50% when the frequency was greater than 35 Hz.

We found reduced impedance when the frequency of electromagnetic field increases (Table 3). The mean impedance of the hybrid and *P.d* between the parental species (Table 3), aligned with the trend in Figure 3. The standard deviation (SD) of impedance followed the same trend as the impedance means among species and hybrids. *P.y* also peaked in the standard deviation of impedance; *P.t* showed the lowest impedance standard deviation, while the hybrid was within the range of *P.t* and *P.y*. The standard deviation of *P.d* was lower than that of the artificial hybrid except at one Hz. Table 3 showed high standard deviations equivalent to individual genotypic differences; thus, the impedance can differentiate intraspecific genetic variation of needles.

Figure 6 depicted the means of each species over the gradient of frequency from zero to 100 Hz and showed the interspecific variation of species. In Figure 5, the positive correlation existed between the standard deviation (SD) and voltage frequency in the hybrid families. However, the SD of *P.y* decreased when the frequency increased. Within the frequency of one Hz to 25Hzthe SD curves of *P.d*, *P.y* paralleled from one to ten Hz. From 20- to 50Hz, SD of *P.d* was between *P.t* and *P.y.* From 45 to 85Hz, *P.d* followed the similar trend as *P.t.* Above 45Hz, the EIS of hybrids diverged from *P.y.* Except for the SD of impedance from one to ten Hz, SD of *P.d*, and the hybrid showed more tendencies of *P.t* than *P.y.* The maternal species characteristic was discernible.

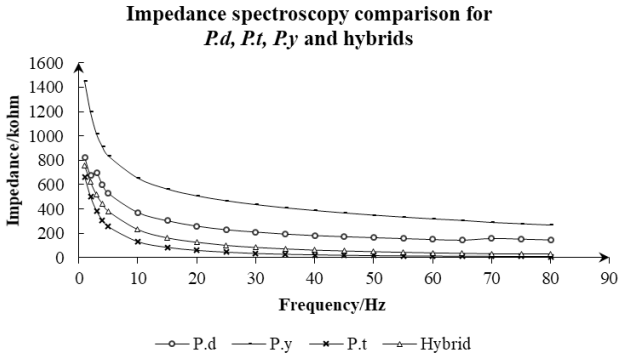

Figure 4. Impedance spectroscopy comparison for *Pinus densata* (*P.d*), *Pinus tabuliformis* (P.t) *Pinus yunnanensis* (P.y), and artificial hybrids

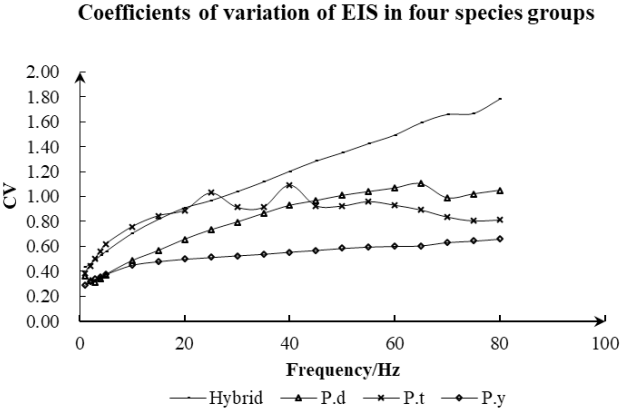

Figure 5. Comparison of the coefficient of variation (CV) of EIS for four species groups

3.5 Interspecific Impedance Models and Cluster Analyses

Per the linear regression of EIS curves of *P.d*, *P.t, P.y* and the hybrids in Figure 1 and 6, there was an exponential relationship between the impedance and voltage frequencies.The curves of each species were following exponential patterns with R^2 greater than 0.9.

The cluster analyses of EIS among *P.d, P.t,* and *P.y* and the hybrids showed that six clusters with the threshold value as 0.4 (Figure 7). There were eight hybrids categorized in the *P.d* cluster indicated the similarity of impedance within the cluster. Hybrids 405 and 413 were clustered with *P.t; P.y* and three hybrids were not classified in the same group of *P.d* or *P.t. P.d* shared similarity with hybrids because the impedance was closer related than to *P.t* or *P.y.*

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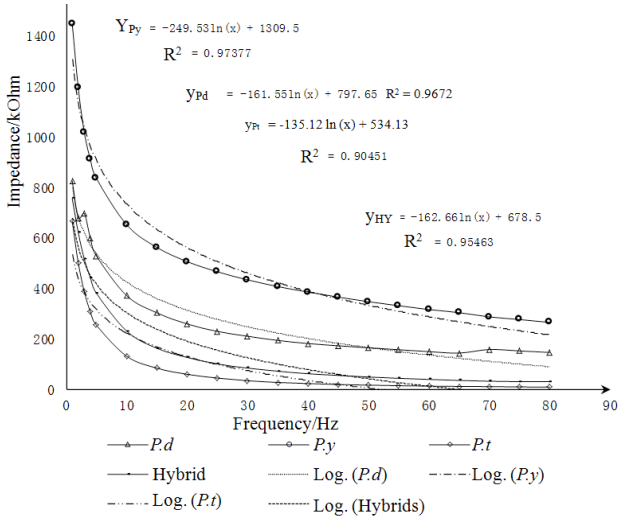

Figure 6. Impedance model of *P.d*, P. t, P.y, and the hybrids

Note:
YPy is the predicted impedance of P.y, the R^2 is 0.97377 which is a good fit; YPd is the predicted impedance of P.d (R^2 = 0.9672); YPt is the predicted impedance of P.t (R^2 =0.9045); YHy is the impedance of the artificial hybrids (R^2 =0.9546).

3.6 Impedance Complex plane

In the complex plane of impedance in the alternating circuits (AC), the real part, resistance (Z'), and the imagery part (reactance, Z'') were both presented. The Nyquist graph [25] depicted both the magnitude of impedance |Z| and the phase difference between the voltage and current in the polar coordinate system (Figure 8).

The artificial hybrid was in the third quadrant; *P.d* entered the fourth quadrant in the high-frequency band, while the rest located in the third quadrant (the entering point is at 12kHz-13kHz). The impedance of *P.t* fell in the fourth quadrant and entered the third quadrant near the eight Hz. The impedance of *P.y* located on the third and the fourth quadrants and the intersection was at 8.5 to nine kHz that bent at 60Hz and formed a closing curve.

The artificial hybrids and *P.d* shared the similar curve, while the curve of *P.d* shifted towards the left. This shift of *P.d* could be the result of genetic variation of needles due to natural selection and adaptation, which needed further studies. The curves of *P.d* and the hybrids tended to be a result of 180 degrees rotation, of that of *P.t* along the Z'' axis. Besides, the curve of hybrids was closer to the shape of the *P.t* curve. The curves of P.d, hybrids and *P.t* showed slower bending at the high-frequency regions, comparing to the steeper bending trends of *P.y* at the high frequency. *P.d* showed similar arcs as *P.y* in the low-frequency area, while *P.d* curve was similar as *P.t* at the high-frequency area.

3.7 Electrical Impedance Evidence of the P.d Hybrid Evolution

Pinus densata has been demonstrated as the ancient homoploid hybrid of *Pinus tabuliformis* and *Pinus yunnanensis*. The EIS revealed the interspecific variability of

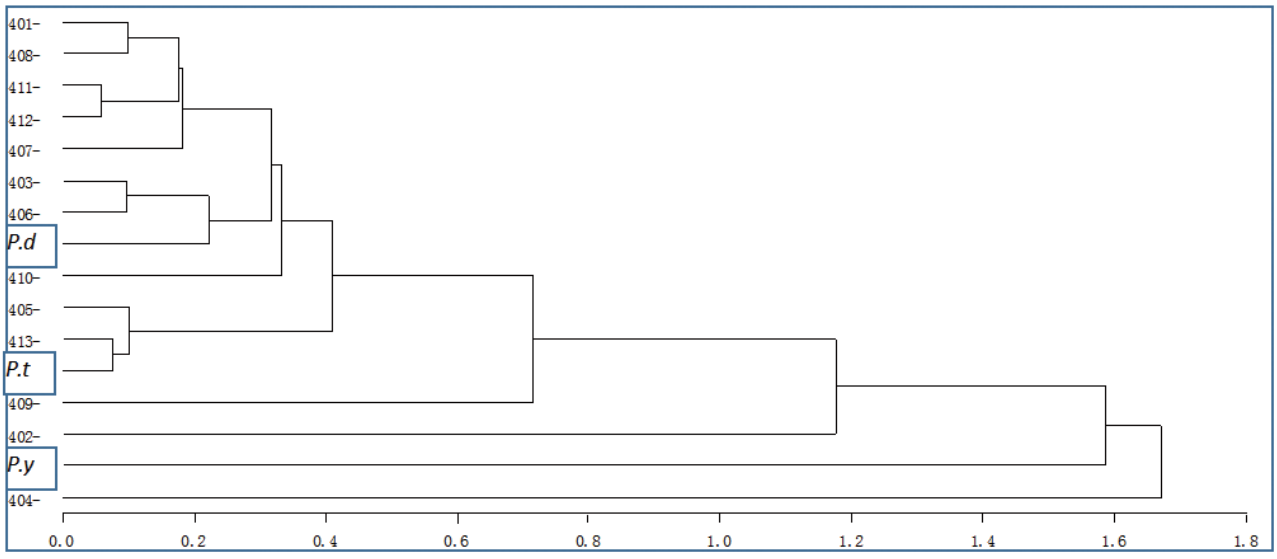

Figure 7. The hybrid combination with P.d and parent species impedance clustering dendrogram (1Hz~80Hz). The x-axis showed the threshold values to form different clusters based on the distance of impedance

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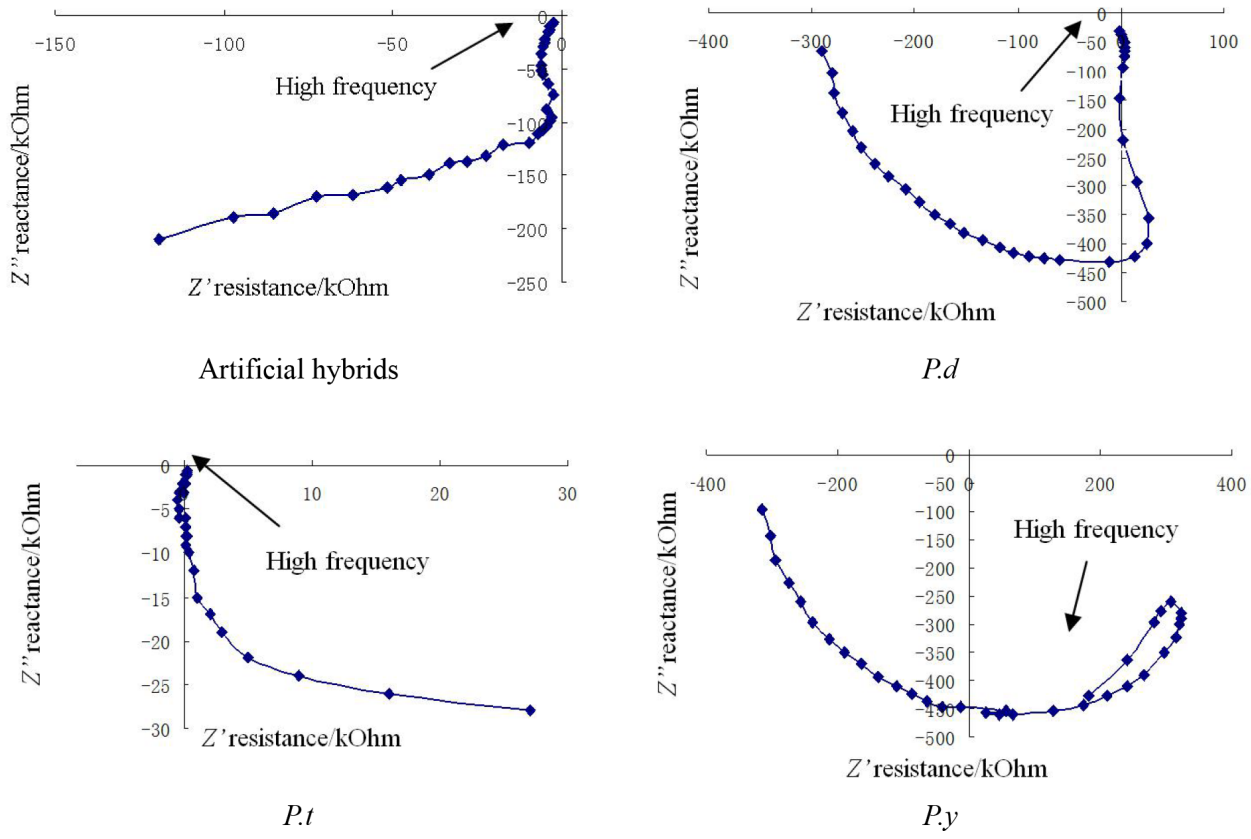

Figure 8. Impedance complex plane for artificial hybrids, Pinus densata (P.d),Pinus tabuliformis(P.t) and Pinus yunnanensis (P.y)

needles that complemented the evolution and physiological relationship between three pure coniferous species. The Nyquistplot showed contrasting trajectories of impedance indicating the specific variability of needles; and the trend of the interspecific genetic variation is aligned with the previous hybridization and evolution findings of *P.d, P.t,* and *P.y* [1,23]. When comparing the germplasms with references of both paternal *P.y* and maternal parent of *P.t* for the hybrids, maternal factor was stronger than the paternal factor for the hybrids. The responses of impedance under different frequencies showed interspecific variations of needles might link to the hybridization history (e.g., Figure 1, Table 2).

P.d adapts to the plateau climate of lower temperature and intense ultraviolet radiation, though the needles of *P.d* and *P.t, P.y* are specialized in terms of adapting to the cold and ultraviolet radiation conditions [7,23]. The nature selection gradient changed the functional and genetic features of needles of the three species [23], though adaptive optimality is more evident in other tree species such as *Pinus contorta* Doug [26] and more studies on the biochemistry of needles is needed.

Contrasting to *P.d,* the parental species *P.y*, and *P.t* are not locally-adapted as *P.d* in Tibetan Plateau. Local adaptation is demonstrated on the morphological and ecological characteristics of the needles, including needle length, the mean number of stomata, mean stomatal density, mesophyll vascular bundle area ratio, mesophyll/resin canal area ratio, mesophyll/(resin canals and vascular bundles) area ratio, vascular bundle/resin canal area ratio etc [23].

Interestingly, the artificial hybrids showed a higher coefficient of variation in impedance (Figure 6) after 40Hz, indicating the hybrids have greater genetic variability in the needle structure comparing to *P.d, P.y,* and *P.t.* The potential heterosis of impedance magnitude occurred among the Hybrids 405, 409, and 403.

The contrast of polar molecules in the needles tissues can be detected by EIS signatures, such as proline. Nonpolar amino acid exists in plants, though their structures contain polar molecules. For an instance, proline has the polar structure as a nonpolar amino acid that can be found freely in plants. Higher concentration of proline was

caused by stress such as drought preconditioning in species such as *Lolium perenne* L. [27], so testing the proline content can evaluate physiological parameters for selecting the drought-resistant varieties. Thus, proline content is a physiological indicator for stress in frost or drought-resistance of plants [28]. Here EIS could detect the signature in normally-grow needles among the studied species. However, the exact relationship of proline concentration in needles between the three coniferous and artificial hybrids needs to be tested in the future studies.

3.8 EIS Reveals Interspecific Variation and Parental Species Effects

The signature of our impedance profiles could describe the interspecific genetic difference of these conifers. *P.y* expressed the highest impedance while *P.t* showed the lowest; impedance of *P.d* and the artificial hybrids were within the range of the two parental species, which agrees with the kinship relations of samples. Xing et al. (2015) reported significant differences in thirteen needle traits between artificial hybrids and *P.y,* the male parent, while the needle characteristics are similar to that of *P.t*(the female parent) and *P.d*. Based on the similarity of needles of *P.d* and artificial hybrids [23], the parental trend is reflected by impedance profile and the cluster analyses.

The EIS under higher frequency (i.e., >40 Hz) indicates more discernable inter-andintra-specific genetic variation (Figure 5). The inter-specific variation is greater than the within-specific variation. Hybrids showed less significant differentiation in impedance, which indicated the limitation of EIS for interspecific studies in pines, though EIS is feasible for interspecific studies.

3.9 Voltage Frequency

The impedance varies greater when the frequency is more than 100Hz. Here we assume the physiological and structure response of needles during the electric stimulus does not modify inter- and intra-specific genetic variation of sampled trees, although the fewer errors of the measurement relies on the controlled and consistent experiment condition settings. More studies are necessary to dissect the effects of physiological and structure response variation under low and high frequency electric stimulus. EIS of the kHz scale could indicate intracellular variation among individual samples based on previous studies [17,21]. Here, we could not exclude the distortions of EI at low frequency (1-80 Hz) due to the potential electrode resistance and excellular resistance.

We found the frequency below 100 Hz is capable of testing needle genotypic variation compared to previous studies focusing on the kHz frequencies [17,21]. The unified regression model has good fitting in low frequency spectrum according to R^2. High frequency at kHz scales show similar trends with moderate to low variations comparing to EI in the low frequency (1-80Hz) in our case (Figure 1 and Supplementary materials). The EIs are linked between the low and high frequency (Supplementary materials).

Potential fractal effects may occur though more studies are necessary. The discrepancy between the low and high frequency also occur in some hybrids (e.g., Hybrid 402). Other than that, EI of hybrids low frequency can still differentiate the genotypic groups.

4. Conclusions

The AC electrical impedance parameters of normal growth needles were measured at a range of frequencie-samong1, 440 needles of 96 trees sampled in 760 seedlings in the habitat of *Pinus densata* in Tibet plateau. We depicted the profile of impedance and constructed the unified regression model, the descent ranking of EIS is *P.y, P.d*, hybrids, and *P.t.* Low voltage frequency(<100 Hz)is capable of demonstrating species variations, while the EIS of high frequency (80Hz-100kHz) showed overlapping trajectories with less interspecific variation. Within the hybrids, family-variation is less than the within-family variation of EIS. The mid-parent and partial maternal species effects of EIS is demonstrated in the hybrid families.

Our non-destructive EIS method is feasible to analyze the genetic relationship between homoploid hybrids, parental conifer, and the artificial hybrids. The EIS pattern not only complemented the previous findings of *P.d* hybrid history, but provided a novel tool for evaluating interspecific variations of conifers due to adaptation differentiation in a non-destructive way.

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Author's Contribution: F-X M lead the study and finished the writing; X-Y C and Y L conceived the ideas and edited the manuscript.

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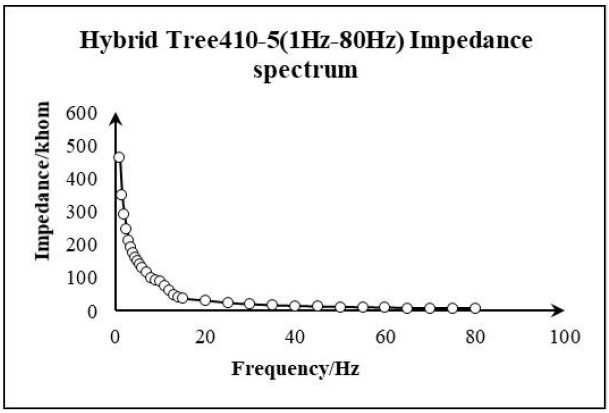
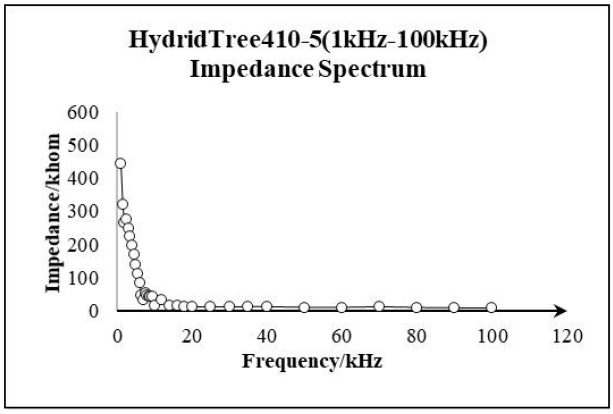
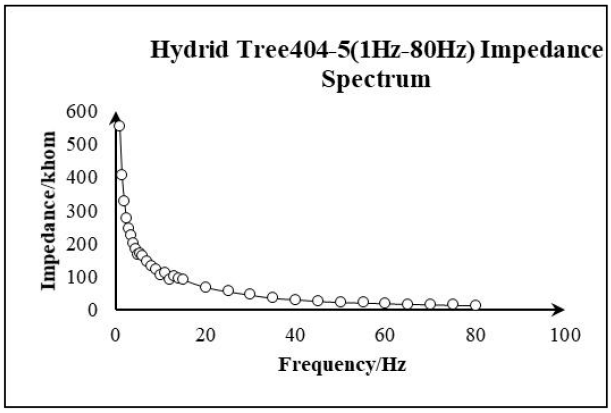
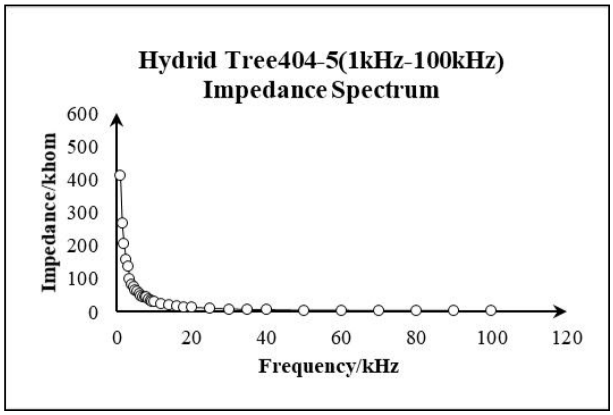
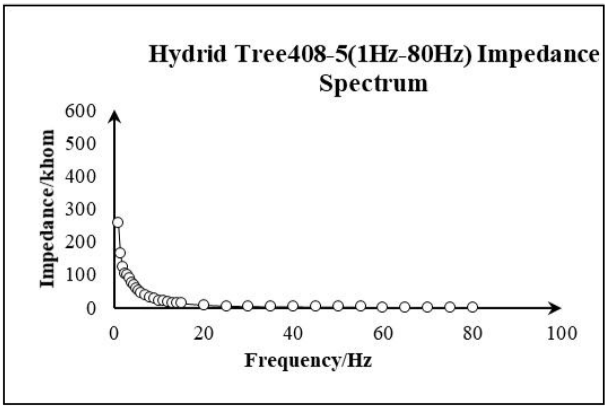
Supplementary Materials

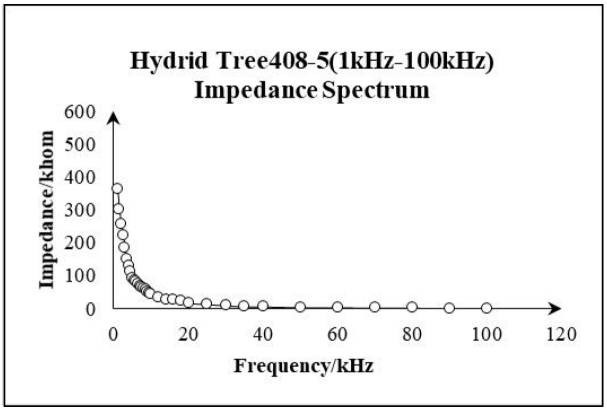


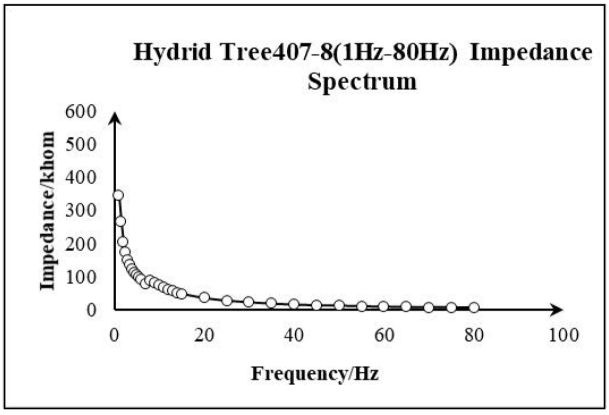


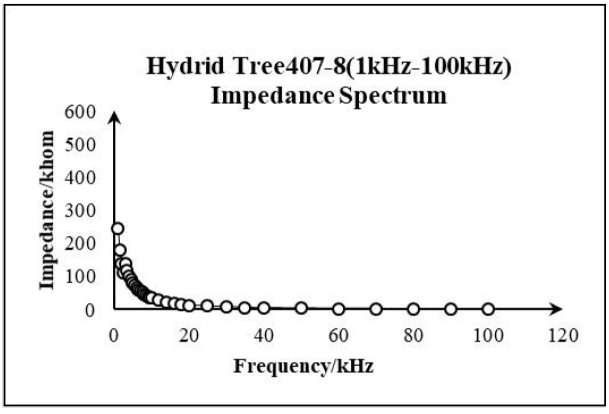












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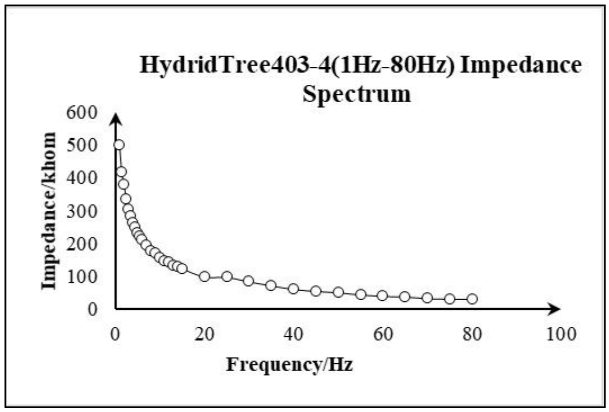

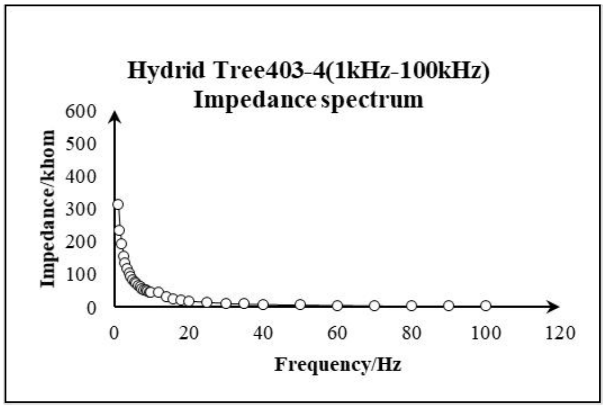

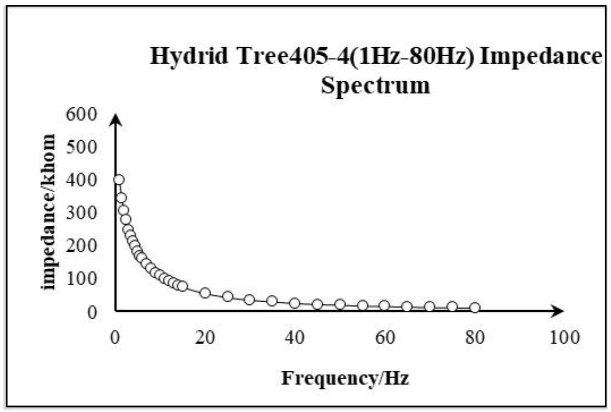

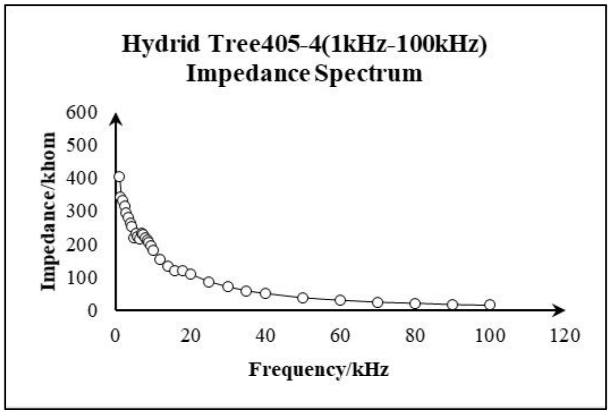

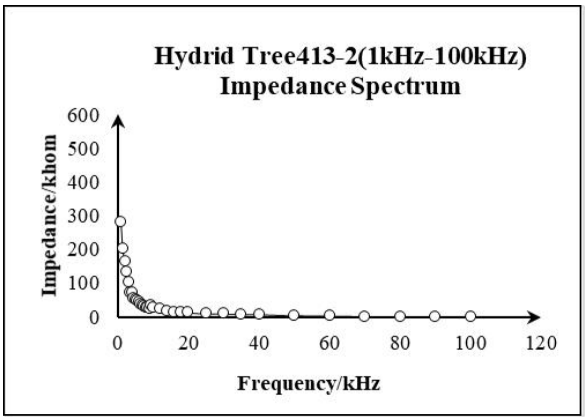

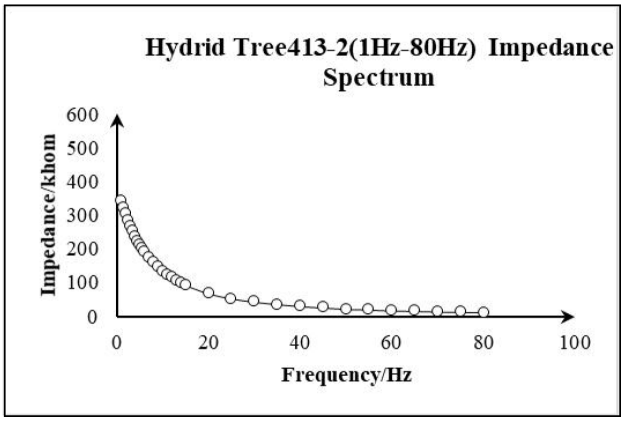

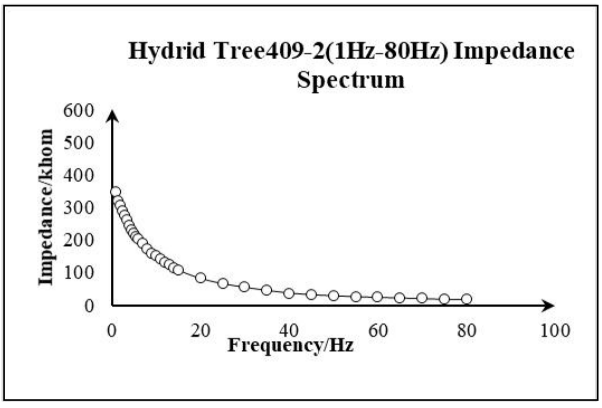

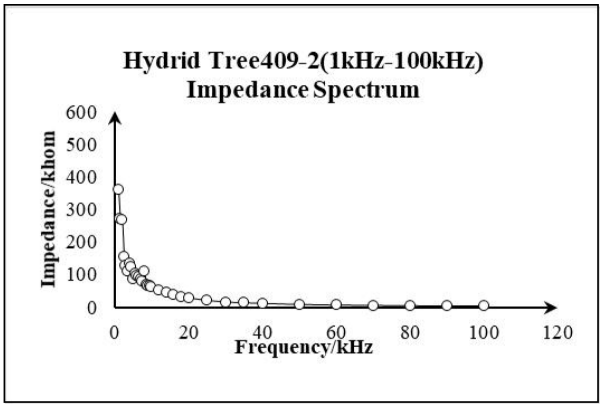

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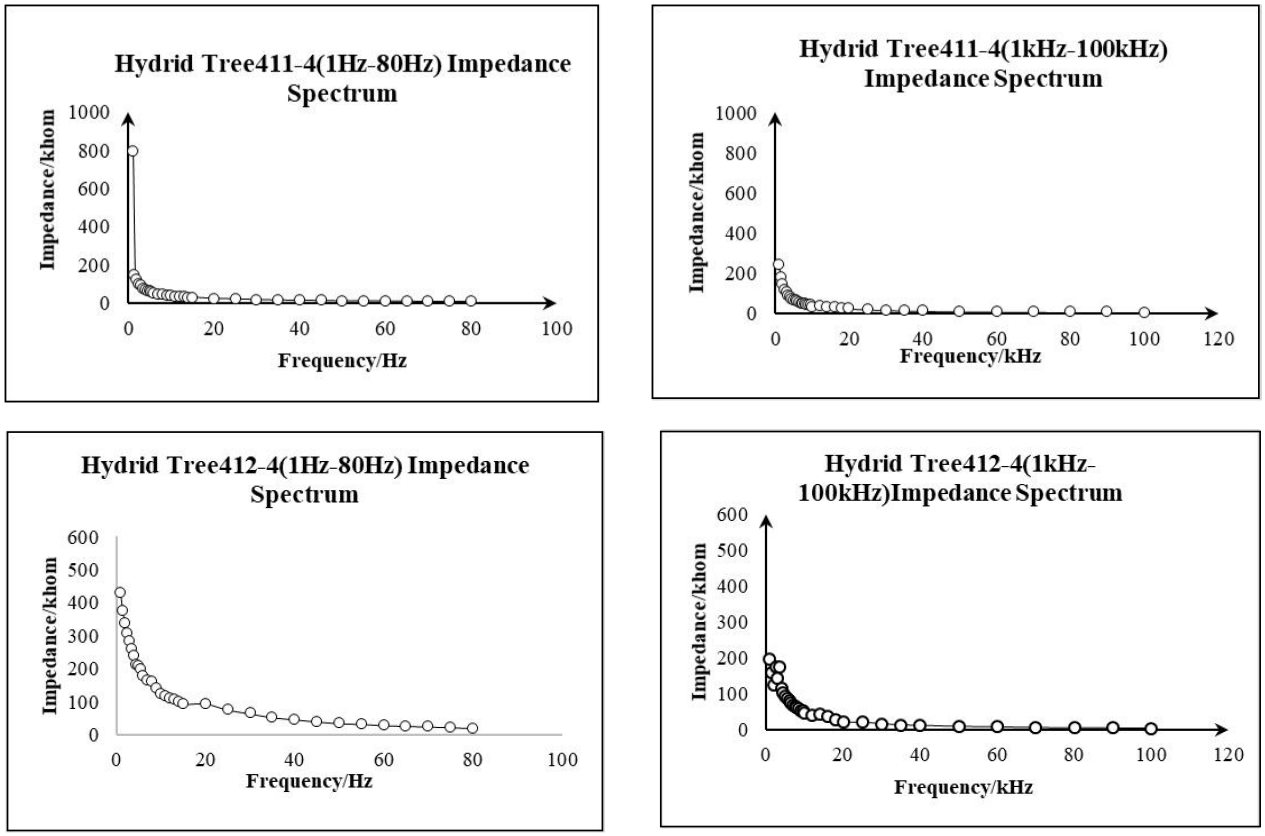
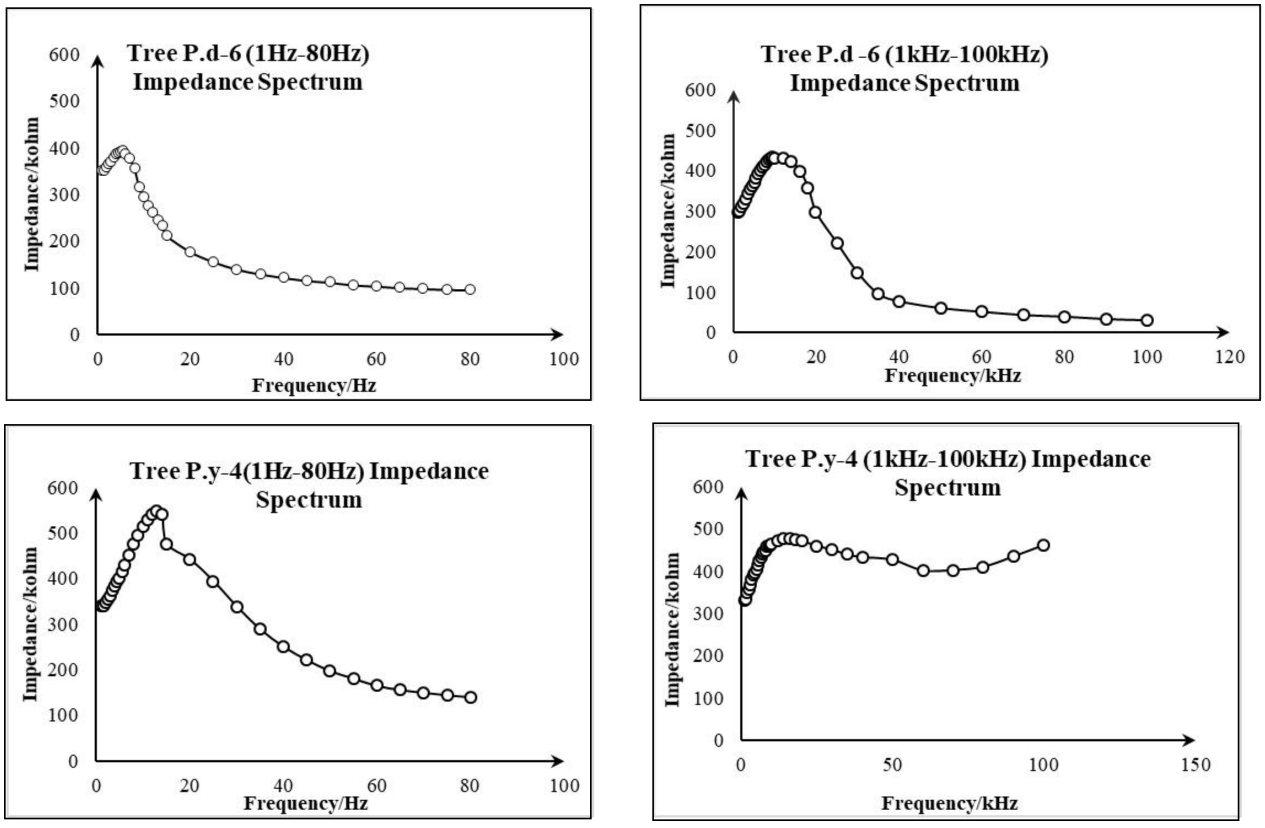


Figure S1. Impedance of hybrids under two spectrums of frequencies, where each curve is the average EI of one sampled tree



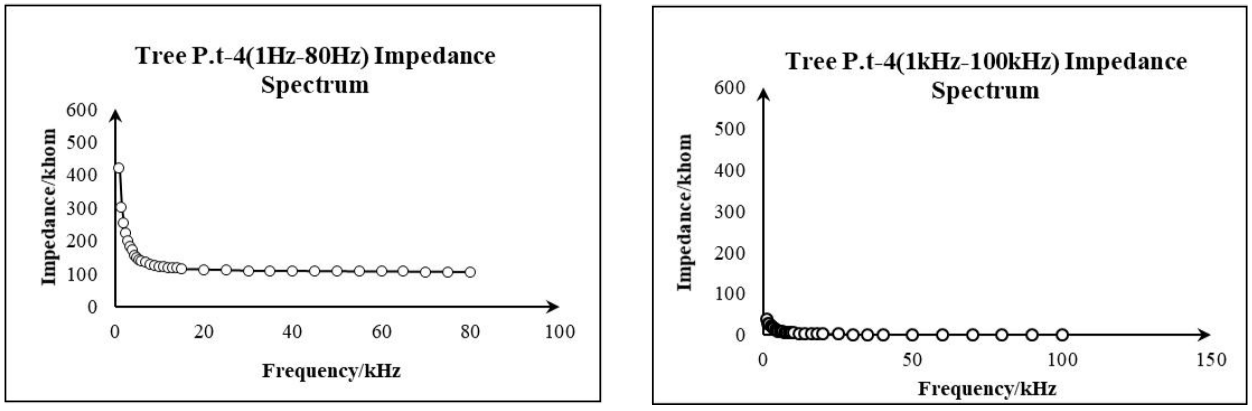


Figure S2. Impedance of *Pinus densata*, P.yunnanensis and P.tabuliformis at different spectrums of frequencies

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ARTICLE

Response of Green Gram (*Vigna Radiata* (*L.*))*Wilczek* to Inter Row Spacing and Phosphorus under Semi-arid Conditions of Eritrea

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ABSTRACT

Green gram (*Vigna radiata* (*L.*) *wilczek*,) commonly known as moong or mung bean or golden gram was introduced to Eritrea as a pulse crop by Ministry of Agriculture at its National Agricultural Research Institute (NARI) in collaboration with Association for Strengthening Agricultural Research in Eastern and Central Africa (ASARECA) in 2012. But its agronomic practices for semi-arid conditions of Eritrea are not yet standardised. Therefore, to find the optimum inter row spacing and phosphorus dose for its higher productivity, a field experiment was conducted at the experimental farm of Hamelmalo Agricultural College, Keren, Eritrea during summer 2015 and 2016. The experiment was conducted in randomised complete block design (RCBD) with 12 treatment combinations of two factors consisting of four inter row spacing (Broadcast, 18 cm, 30 cm, and 45 cm) and three phosphorus levels (0, 20 and 40 kg P_2O_5 ha^{-1}) each replicated thrice. The results of the study revealed that sowing of K-26 bold seeded variety either by broadcast method or at 18 cm inter row spacing at10 cm plant to plant spacing fertilized with 40 kg P_2O_5 ha^{-1} through DAP fertilizer drilled at the time of sowing proved significantly superior to increase growth, yield attributes and seed yield of green gram.

1. Introduction

Legumes are cultivated in the highlands and mid lands of Eritrea covering about 20,000 ha of land annually [1]. Faba bean, chick pea, grass pea, lentil, cowpea and field pea are the common legumes that are widely grown in Eritrea and are the part of main diet of the Eritrean society. They are mostly used as main dish (*shiro*), snacks (*titko or migo*), fresh green (*shewit*), and roasted (*kolo*) and bread (*kicha*). The inexpensive and high-quality plant protein that legumes possess makes them important substitutes to the expensive animal product proteins.They exhibit good drought resistance and can be an option in marginal areas where other cereal crops cannot be planted. This makes them potentially very valuable for crop diversification in low rainfall conditions.

Among the pulses, Green gram (*Vigna radiata (L.) Wilczek*) also called as mung bean or golden gram is considered as a poor man's meat containing almost double amount of protein as compared to cereals. It has good digestibility and flavor. It contains 62.62% carbohydrate, 23.86% protein, 1.15% fat, 3.7 % ash, 48 mg vitamin C, 132 mg calcium, 367 mg phosphorus, 1246 mg potassi-

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um, 15 mg sodium, 18.6 mg magnesium and 1450 kJ Energy[2], apart from thiamin, riboflavin, niacin vitamins and special amino acids like tryptophan, lysine, methionine, leucine, and isoleucine. Hence, from the nutritional point of view, mungbean is perhaps the best among all other pulses. In addition to this it also improves the soil fertility through biological nitrogen fixation to the tune of 30-40 kg N ha^{-1}[3] which plays a vital role in sustainable agriculture.

Green gram was introduced in Eritrea by the Ministry of Agriculture at National Agricultural Research Institute (NARI) in collaboration with the Association for Strengthening Agriculture Research in Eastern and Central Africa (ASARECA) in 2012. It was given for cultivation to the farmers of Zoba Anseba and Gash Barka as inter crop with Sorghum and Pearl millet. The production of this crop was observed to be good and motivated farmers to grow this legume as intercrop because it is drought tolerant, early maturing and therefore it secures against total crop failure. But due to lack of package of agronomic practices for its cultivation under local agro-climatic conditions, its potential as a sole or intercrop has not yet been exploited in Eritrea.

Among the various agronomic practices, optimum nutrient management and plant population are important for increasing productivity of green gram. Out of the major nutrients, phosphorus is considered as the key element for obtaining the higher yield of legume crops, as it plays an important role in process of photosynthesis, root formation, growth, development, seed yield and enhancement of maturity of the crop. Phosphorus also reduces the harmful effects of excess nitrogen and imparts resistance to crop plants against diseases. Supply of phosphorus to legume increase the number and size of root nodules and nitrogen fixing potentiality of rhizobium, [4, 5]. [6] found that phosphorus application up to 60 kg P_2O_5 ha^{-1} increased both the dry matter accumulation and seed yield of mung bean. Similarly optimum plant populations which can be manipulated by inter or intra row spacing contribute to the high yield because thick plant population will not get proper light for photosynthesis and is easily attacked by diseases. On the other hand very thin population will not be able to fully utilize the resources and thus reduce the yield [7]. [8] reported that row spacing of 20 and 30 cm and plant spacing of 5 and 10 cm resulted in significantly higher seed yield of green gram compared to 50 cm row spacing and 15 cm plant spacing during both summer and rainy seasons.

Green gram being a new crop in this country no systematic research has been done on its method of sowing and phosphorus requirement as a sole or intercrop. Therefore, keeping in view the above facts in mind, the present investigation was conducted to standardise the row spacing and phosphorus level for higher productivity of green gram under semi-arid conditions of Eritrea.

2. Materials and Methods

The field experiment was carried out at the experimental farm of Hamelmalo Agricultural Collage, during summer 2015 and 2016. The site is located in Zoba Anseba of Eritrea at 15^0 52' 18'' N latitude, 38^0 27' 55'' E longitude and altitude of 1280 meter above mean sea level. The area experiences a mean annual rainfall of 513.5 mm, maximum temperature of 34.7^0C and minimum temperature of 11.1^0C. It falls under semi-arid mid-land region of Eritrea.

The soil of the experimental field was sandy loam in texture with moderately alkaline pH (8.07), low in total N (0.05 %), very low in Phosphorus (2.04 ppm), low in Potassium (0.18 cenmol/kg) and with electrical conductivity of 0.13dsmol/m.

The bold seeded variety K-26 of green gram, which was introduced to Eritrea in the year 2012, was used in the present experiment. The field experiment was conducted in a Randomized Complete Block Design (RCBD) with three replications and 12 treatment combinations of two factors consisting of three phosphorus levels (0, 20 and 40 kg P_2O_5 ha^{-1}) and four inter-row spacing (broadcast (farmers method), 18 cm, 30 cm and 45 cm). The treatments were allocated randomly to gross plots of size 4m x 2.7m in each of the three replications after proper seedbed preparation and experiment layout manually. Crop was sown manually on July 9 and July 2 during 2015 and 2016, respectively by keeping inter row spacing as per treatments and uniform plant to plant spacing of 10 cm. In broad cast treatment, seed equal to the quantity used in 18 cm row spacing was broadcasted and mixed in upper 7 cm soil layer on the prepared seedbed. Phosphorus was applied as per the treatments by using di-ammonium phosphate (DAP) fertilizer. The 16 kg N ha^{-1} supplied by 40 kg P_2O_5 ha^{-1} through DAP was equalized in all other treatments by applying required amount of urea. Thinning was done at 15 days after sowing (DAS) to maintain the recommended plant density per plot. First weeding was done at the time of thinning and second weeding was done at 35 DAS. Five plants in each plot were randomly selected to record the data on different growth, development, nodule count and yield attributes. The weed count data were recorded by placing 50cm x 50cm quadrate at random in each plot for 15 days after the second weeding. The samples taken for dry matter were oven dried at 70 ^{0}C until constant weight was achieved.

Crop was harvested when about 80% of the pods be-

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came black from 6.93 m^2, 6.93 m^2, and 7.35 m^2 and 6.3 m^2 net plot area in plots having broadcast, 18 cm, 30 cm, and 45 cm treatment combinations, respectively by keeping one border row and 25 cm from each side of the plot as non-experimental area. Seed yield and biological yield were recorded from the net plot area and converted to per hectare. The harvest index was calculated by dividing the seed yield by biological yield per plot.

The data obtained were statistically analysed in RCBD design using Analysis of Variance (ANOVA) with the help of GENSTAT 14 statistical computer package software at 5% level of significance.

3. Results and Discussion

The weather conditions during first year of the experimentation were comparatively more favorable than second year for the growth, development and yield of the crop because of receipt of higher and uniform distribution of rainfall (302.5 mm) as compared to second year when only 235 mm of rainfall was received. As a result comparatively higher seed yield of green gram was obtained during the year 2015 as compared to the year 2016.

The results emanated in two years' studies have been presented in Tables 1-5 and discussed with cause and effect relationships.

3.1 Effect on Growth

The data on the effect of row spacing and phosphorus levels on plant height, dry matter, effective nodules and weed count have been presented in Table 1. The data reveal that row spacing did not influence the plant height and dry matter per plant of green gram significantly during both the years of experimentation. Effective numbers of nodules per plant were not significantly affected by the row spacing during both the years.

Row spacing influenced the weed population significantly during the first year but the differences were not significant during the second year. During the first year broadcast and 18 cm row spacing being statistically at par reduced the weed population significantly over 30 cm and 45 cm row spacing due to the smothering effect of higher mung bean population and early canopy formation in narrow row spacing.

Phosphorus levels did not significantly influence plant height, dry matter and nodule count during both the years of study. However, addition of phosphorus numerically increased the plant height, dry matter and nodulation over no phosphorus application. Weed population was not affected significantly by phosphorus levels during both the

Table 1. Effect of treatments on growth parameters, effective nodules and weed count

Treatments	Plant height (cm)		Effective nodules (No. plant^{-1})		Dry matter (g plant^{-1})		Weed count (No. m^{-2})	
Row spacing(cm)	2015	2016	2015	2016	2015	2016	2015	2016
Broadcast	43.4	44.7	4.0	3.2	166.0	16.8	2.9	10.4
18	44.0	38.8	3.8	3.3	150.0	16.1	3.3	11
30	46.4	40.1	3.8	3.4	162.8	15.9	4.8	9.7
45	41.1	44.6	3.6	4.6	177.9	19.2	6.0	10.7
LSD (0.05)	NS	NS	NS	NS	NS	NS	0.88	NS
Phosphorus levels (kg ha^{-1})								
0	42.8	39.8	3.7	3	157.5	16.5	3.9	10.7
20	44.9	43.9	3.4	3.5	169.2	16.9	4.1	10
40	43.6	40.9	4.3	3.6	166.3	17.7	4.8	10
LSD (0.05)	NS	NS	NS	NS	NS	NS	NS	NS
CV%	10.2	13.9	13.5	44.7	25	19.4	21.5	11.7
Row spacing x Phosphorus levels	NS	NS	NS	NS	NS	NS	NS	NS

NS=Non-significant

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Table 2. Effect of treatments on development of green gram

Treatments	Days taken to flowering		Days taken to maturity	
Row spacing(cm)	2015	2016	2015	2016
Broadcast	47.4	42.1	69.7	66.2
18	42.3	42.1	64.3	66.4
30	46.2	42.6	68.8	67.2
45	46.6	42.1	67.9	68.7
LSD (0.05)	3.32	NS	3.66	NS
Phosphorus levels (kg ha^{-1})				
0	47.5	42.6	69.3	67.4
20	45.4	42.1	67.3	67.3
40	44.0	42	66.4	66.7
LSD (0.05)	NS	NS	NS	NS
CV%	5.5	2.3	7.5	3.5
Row spacing x Phosphorus levels	NS	NS	NS	NS

years of experimentation.

The row spacing and Phosphorus levels did not interact significantly to influence the growth, nodulation and weed count during both the years of experimentation.

3.2 Effect on Development

The data on the effect of different treatments on days taken to flowering and maturity of green gram have been presented in Table 2. A perusal of the data indicate that row spacing did not influence the time taken for flowering and maturity of green gram during the second year but during the first year row spacing caused significant influence on time taken to flowering and maturity. Sowing at 18 cm row spacing resulted in significantly earliest flowering and maturity of the crop over other row spacings.

Phosphorus levels did not influence the time taken to flowering and maturity of the crop during both the years. However, numerically increasing phosphorus levels caused comparatively earliness in flowering and maturity of the crop during both the years.

On an average the crop took 42-45 days and 65-68 days for flowering and maturity, respectively which helped it to escape the moisture stress due to withdrawal of rains in later part of the crop development.

Row spacing and phosphorus levels did not interact significantly to influence the days taken for flowering and maturity of the crop.

3.3 Effect on Yield Attributes

The data on effect of treatments on yield contributing characters have been presented in Table 3. A critical perusal of the data reveal that effective plant population, number of pods/plant, number of branches /plant were significantly affected by row spacing during both the years of experimentation. However, 1000 seed weight was not affected significantly during both the years. Although increase in row spacing increased the number of pods per plant and number of branches per plant significantly due to mutual competition free environment, but the effective plant population decreased significantly which ultimately reduced the seed yield of green gram with increased row spacing (Table 4). These findings are in agreement with those of [9] who also reported an increase in number of pods per plant with lower plant population of mung bean.

Further perusal of the data in Table 3 reveal that phosphorus levels did not influence 1000 seed weight and number of pods significantly during both the years, effective plant population during the first year and number of

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Table 3. Effect of treatments on yield contributing characters of green gram.

Treatments	Effective plant population (No.m^{-2})		1000seed weight(g)		No. of pods (No. plant^{-1})		No. of branches (No. plant^{-1})	
Row spacing(cm)	2015	2016	2015	2016	2015	2016	2015	2016
Broadcast	26.6	30.9	54.8	51.9	21.0	23	8.2	6.2
18	49.2	51.8	53.7	53.8	17.0	21.9	7.8	5.9
30	27.8	30.0	53.9	53.6	23.0	27.4	9.7	7
45	17.3	20.9	52.8	56.0	26.0	32.4	10.2	8
LSD (0.05)	2.94	1.68	NS	NS	3.35	0.60	0.97	0.30
Phosphorus levels (kg ha^{-1})								
0	30.6	32.6	54.4	53.7	19.9	25.9	8.4	6.5
20	30.9	33	52.9	53.8	22.3	25.8	9.6	6.5
40	29.6	34.5	54.0	53.9	23.3	26.9	8.9	7.3
LSD (0.05)	NS	1.45	NS	NS	NS	NS	0.84	NS
CV%	9.9	5.1	10.2	9.1	15.7	23.8	11.1	16.7
Row spacing x Phosphorus levels	NS	NS	NS	NS	NS	NS	NS	NS

NS= Non-significant S= Significant

Table 4. Effect of treatments on seed yield, Stover yield, biological yield and harvest index of green gram.

Treatments	Seed yield (kg ha^{-1})		Stover yield (kg ha^{-1})		Biological yield (kg ha^{-1})		Harvest index (%)	
Row spacing(cm)	2015	2016	2015	2016	2015	2016	2015	2016
Broadcast	2016	1533	3564	2763	5750	4296	35.1	35.7
18	2281	1358	3669	2284	6116	3642	37.3	37.3
30	1918	1136	3467	2083	5541	3186	34.6	34.6
45	1747	1124	3446	2116	5141	3240	34.0	34.7
LSD (0.05)	317.2	205.1	NS	111.4	NS	327.5	NS	NS
Phosphorus levels (kg ha^{-1})								
0	1990	1177	3598	2066	5988	3382	33.2	34.8
20	1977	1241	3584	2138	5277	3666	37.5	33.9
40	2004	1447	3426	2048	5646	3802	35.5	38.1
LSD (0.05)	NS	117.6	NS	96.5	NS	NS	NS	NS
CV%	16.3	16.4	20.9	20.3	18.7	18.5	10.5	12.5
Row spacing x Phosphorus levels	NS	S	NS	NS	NS	NS	NS	NS

NS= Non-significant S= Significant

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branches per plant during second year of experimentation. However, pods per plant increased numerically with the increase in phosphorus levels during both the years. [10] also reported a similar increase in number of pods /plant with increase in phosphorus levels .Significantly highest effective plant population was obtained with 40 kg P_2O_5 ha^{-1} during the second year of experimentation. During the first year of study 20 kg P_2O_5 ha^{-1} being statistically at par with 40 kg P_2O_5 ha^{-1} produced a significantly higher number of branches per plant over no phosphorus application.

Row spacing and phosphorus levels did not interact significantly to influence the yield attributes of the crop during both the years.

3.4 Effect on Yields

The data regarding the effect of treatments on biological yield, stover yield, seed yield and harvest index of green gram have been presented in Table 4. A cursory glance at the data reveals that while row spacing significantly influenced the biological yield and stover yield only during the second year of experimentation, and the seed yield was influenced significantly during both the years of study. However, row spacing did not influence the harvest index significantly during both the years. During the first year of the study, biological and stover yields were not influenced significantly by row spacing. But during the second year, broadcast method of sowing resulted in significantly highest biological and stover yield which may be ascribed to the higher and non-uniform total plant population but lower effective plant population. Row spacing of 18 cm was the next best because of higher effective plant population (Table 3) as well as total plant population of green gram and lower weed count (Table 1) as compared to other row spacing. These results are in agreement with those of [11] who also obtained higher biological yield at narrow row spacing of 20 cm as compared to 43 row spacing. Row spacing of 18 cm being statistically at par with broadcast method of sowing resulted in significantly higher seed yield of green gram over other row spacings. It may be ascribed to the efficient utilization of light, moisture, nutrients and space by the plant population at 18 cm inter row spacing resulting in comparatively higher effective plant population and 1000 seed weight (Table 3).The row spacings of 30 cm and 45 cm being statistically at par were next best in seed yield because of low effective plant population, but a higher number of pods and branches per plant (Table 3) during both the years of experimentation. These results are in agreement with those of [8] who also reported higher seed yield of green gram at 20 cm and 30 cm row spacings compared to 50 cm row spacing. These findings are further in conformity with those of [12] who recorded 15% higher seed yield of green gram at 20 cm row spacing over 30 cm row spacing. Although the harvest index was not influenced significantly by the row spacings but numerically highest harvest index (37.3) was obtained with 18 cm row spacing during both the years.

A perusal of data in Table 4 further revealed that phosphorus levels under study did not significantly influence the biological yield, stover yield and harvest index of green gram during both the years and the seed and stover yield during the first year of experimentation. However, during second year 40kg P_2O_5 ha^{-1} produced significantly highest seed yield over 0 and 20 kg P_2O_5 ha^{-1} which is attributed to higher effective plant population, 1000 seed weight, number of pods and branches per plant (Table 3) at higher level of phosphorus. This finding is in corroboration with those of [13] who obtained higher seed yield of green gram at 30 to 60 kg P_2O_5 ha^{-1} and of [14] who reported the highest seed yield at 40 kg P_2O_5 ha^{-1}.

Biological yield, stover yield and harvest index of green gram were not affected significantly by the interaction of row spacing and phosphorus levels during both the years. However, seed yield was influenced significantly by their interaction during the second year of experimentation.

Table 5. Interaction effect row spacing and phosphorus levels on seed yield (kg ha^{-1}) of green gram during 2016.

	Phosphorus levels (kg ha^{-1})		
Row spacings (cm)	**0**	**20**	**40**
Broadcast	1316	1528	1754
18	1315	1249	1512
30	1002	1102	1305
45	1076	1083	1215
LSD (0.05)			355.2

A critical perusal of the row spacing and phosphorus interaction data in Table 5 reveal that although in each of the row spacing, increase in phosphorus levels increased the seed yield numerically, but significantly highest increase in seed yield (1754 kg ha^{-1}) was obtained with a combination of 40 kg P_2O_5 ha^{-1} and broadcast sowing. However, it was statistically at par with combination of 18 cm row spacing and 40 kg P_2O_5 ha^{-1}(1512 kg ha^{-1}) and broadcast sowing with 20 kg P_2O_5 ha^{-1}(1528 kg ha^{-1}). These results are in agreement with those of [15,16] who reported that 40 kg P_2O_5 ha^{-1} and 30 cm row spacing gave highest seed yield of 701.1 kg ha^{-1} and 860 kg ha^{-1}during first and second year

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of study, respectively in sandy loam soils of India.

4. Conclusions

It can be concluded from the study that growing of green gram by broadcast method or 18 cm row spacing and drilling of 40 kg P_2O_5 ha^{-1} through di-ammonium phosphate (DAP) at the time of sowing resulted significant increase in growth, yield attributes and seed yield of green gram under semi-arid conditions of Eritrea. However, for getting uniform distribution of plant population and ease of intercultural operations, sowing in rows at 18 cm row spacing should be preferred over broadcast method of sowing.

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ARTICLE

Soybean Improvement for Lipoxygenase-free by Simple Sequence Repeat (SSR) Markers Selection

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ABSTRACT

Beany flavor of soybean (*Glycine max* (L.) Merr.) is caused by oxidation of polyunsaturated fatty acids by the action of three lipoxygenases (LOX1, LOX2 and LOX3) present in mature seeds. The unpleasant flavor restricts human consumption of soybean products. This problem could be solved through genetic elimination of alleles that code these enzymes. Parental cultivars and two hybrid population were selected and analyzed using genetic markers for alleles locus, encoding Lox_1, Lox_2 and Lox_3 free. The SSR marker Satt 212 confirmed the presence of the homozygous null-allele Lx_3 in the cultivar BRS 213, which were used for hybridization with BR 36. Heterozygote F_1 hybrid plants and homozygous Lx_3 lines in F_2 segregating populations were successfully identified. The SSR markers Sat090 and Sat417 were the most effective diagnostic markers among all SSR markers tested. Satt090 and Satt417 confirmed the presence of the homozygous Lx_2 null-allele in the parental cultivar BRS 213 by flanking Lx_2 loci at 3,00 and 2,77 cM, respectively. The presence of Lx_2 null allele in the F_2 segregating populations between BRS 213 and BRS 155 was successfully identified with a selection efficiency of 98% and have great potential for further application in the Brazilian breeding program aimed at improving soybean seed quality.

1. Introduction

Soybean is the cheapest source of protein with high quality; in addition, it has a low content of saturated fat. Also due to these properties, it is commonly used in the diet of many populations worldwide. Soybean contains several special bioactive ingredients, such as tocopherols, isoflavones, saponins, among others, that can contribute to prevent chronic diseases such as cancer, atherosclerosis, diabetes, Parkinson's, etc. Recently, soy-containing foods have gained approval and are considered as the "functional

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food" of this age.

Health-conscious consumers have changed on eating habits and preferences especially in the middle and upper classes. The population aging and the quest for a healthier life have promoted the use of soybean as an alternative to the human diet because of its contribution to reduce the incidence of cardiovascular disease. This has determined an increase of soybean request and consumption for food use worldwide. As a consequence, the market of soybean cultivars with higher agronomic characteristics and seed composition traits has been growing and cultivars of food type are on the demand.

Brazil is the second largest producer with 115.072, 5 million tons produced in 2019, behind only the United States with 123,664 million tons [1]. However, less than 5% of the total soybean produced in Brazil is used for soy foods. The reason is due to the physiology process after post-harvest that affects the seeds with high lipoxygenase (LOX) activity and linoleic acid content with a consequent production of hexanal that even at low concentrations (e.g. 5 ppb), confers unpleasant odor and therefore flavor; this makes difficult the production of soybean-containing food with suitable properties for human consumption. Since lipoxygenase (linoleate: oxygen oxidoreductase EC 1.13.11.12) mediated oxidation of polyunsaturated fatty acids in mature soybean seeds results in unpleasant odor and flavor, lipoxygenase-free seed soybean cultivars specially developed for human consumption can contribute to increase the acceptability of soy foods, for this reason they are becoming important to the food industry and are in demand. The major reason of this off-flavor is the aldehydes and ketones produced by the activity of an iron containing dioxygenase that catalyzes the oxidation of unsaturated fatty acids [2].

Heat treatment has been used commercially to suppress the lipoxygenase activity in order to prevent the beany-flavor generation in soy protein products. However, heat treatment sufficient to inactivate the lipoxygenase action often results in the insolubilization of the soy proteins, generating an unpleasant "cooked" odor. In addition, soybean cultivars lacking the lipoxygenases should become economically valuable due to their enhanced storage stability, since the lipoxygenase-induced oxidative deterioration of protein and oil in soy meals is reduced. A combination of appropriate processing technologies and the new cultivars may enable the production of various soybean-based foods.

There are three major aspects of plant physiology where lipoxygenases have been implicated: growth and development, senescence, and insect wound resistance response and pest resistance. However, it has been demonstrated that lipoxygenase-free seeds regularly develop into normal plants without defects, independently from the role that this enzyme has in the plant physiology [3, 4].

Lipoxygenase is present in mature soybean seeds in the form of three isozymes i.e., lipoxygenase-1 (LOX1), lipoxygenase-2 (LOX2) and lipoxygenase-3 (LOX3). They are encoded by three dominant genes which are inherited in the mendelian way. Functional genes for the isozymes have been assigned and the gene symbols are: Lx_1, Lx_2 and Lx_3. The inheritance and molecular base of lipoxygenase nulls have been investigated in different backgrounds for several authors [5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15]. Spontaneous mutants of the enzymes have been identified and several mutants were developed by gamma radiation [16, 17]. The Lx_1 and Lx_2 loci were found to be in tight genetic link and mapped in the same linkage group, while Lx_3 locus is independent [7].

The development of seed lipoxygenase-free triple null mutant genotype was reported by Hajika et al. [16] and Kitamura [18]. More recently Wang et al. [19] have reported that triple null mutant plants carrying *gmlox1gmlox2gmlox3* triple mutations were generated by clustered regularly interspaced short palindromic repeats (CRISPR)-CRISPR associated protein 9 (Cas9). The authors described that the original repulsion-phase linkage in independent sources of mutant alleles at the Lx_1 and Lx_2 loci was broken, resulting in a coupling-phase linkage that eventually led to the development of a triple null lipoxygenase genotype. However, there were no data about the mechanism that generated the genotype without the three seed lipoxygenases.

Accurate evaluation assays are necessary if the appropriate genotype has to be selected for the development of seed lipoxygenase free soybean cultivars. Laborious and time-consuming seed phenotype methods have been used to determine the presence of lipoxygenase in breeding programs. In addition, individuals that are homozygous and heterozygous in the same generation cannot be discriminated using these procedures. In contrast, a screening procedure based on codominant DNA markers developed for lipoxygenase nulls mutation could be used in breeding programs for market-assisted selection (MAS) of lines with seed lipoxygenase nulls. Microsatellite or simple sequence repeat (SSR) marker is a sequence with one to six base pairs repeated in tandem. SSRs consist of small repeat units (1-6 bp) distributed in tandem throughout the genomes and are easily detected by polymerase chain reaction (PCR); they are relatively abundant, have extensive genome coverage, and require a low amount of DNA for amplification, there are 874 SSR markers assembled in ordered positions within the 20 soybean linkage groups. Thus, they have the potential to accelerate and simplify

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breeding efforts for soybean cultivars with improved flavor [13, 14, 15, 20, 21, 22].

This study was undertaken to evaluate the efficiency of microsatellite markers, previously identified as being associated with free-lipoxygenase in Brazilian soybean cultivars lacking seed lipoxygenases to be used as germplasm resource in breeding program focused on selecting cultivars with improved flavor.

2. Material and Methods

2.1 Plant Material and Development of Segregating Population

The plants were grown at greenhouse at Agronomy Department of State University of Londrina, Londrina – PR, at 23º22' latitude south, the phenotyping and genotyping tests were performed at the Laboratory of Biotechnology of Embrapa Soybean, Londrina - PR. $F_{2:3}$ derived populations of 168 RILs were developed by single seed descent from BRS 213 X BR 36 and $F_{2:3}$ derived population from 93 RILs from BRS 213 x BRS 155. Seeds of parental cultivars were obtained from Active Germplasm Bank of Brazilian Agricultural Research Corporation (Embrapa Soybean). The cultivars were chosen for their special characteristics for human consumption. BRS 213 (BR 94-23354 X BR 94-23321) is triple null for lipoxygenase isoenzymes. It was released in 2002 making available excellent raw materials for soy food processing uses, such as soymilk and tofu, due to its lack of beany taste. BR213 has as ancestral the Japanese line triple null seed lipoxygenases (obtained from Dr. Kitamura - National Agricultural Research Centre, Ministry of Agriculture, Forestry and Fisheries, Tsukuba, Japan). The triple null mutant soybean line was produced by gamma-ray irradiation of F_2 plants from a cross between two double mutants: Lx_1- and Lx_3-free and Lx_2- and Lx_3-free [16, 18].

The Brazilian cultivar BR 36 (IAS 4(2) X BR 78-22043) is dominant for the three seeds lipoxygenase genes (Lx_1, Lx_2, Lx_3). It has as clear hilum color, large grains, high protein content and good sensory qualities, which give grains, flour and milk with mild flavor. It is a cultivar preferred by organic growers and it is widely accepted in the soybean market for human [23]. BRS 155 (IAS 4(2) X PI 157440) is a Brazilian cultivar that was released for soy food uses, it has the allele *kti* from the ancestral cultivar Kin-du (PI 157440), a South Korean cultivar that has the *kti* allele. The dominant gene KTI controls an anti-nutritional factor of trypsin inhibitor Kunitz that affects protein digestibility [24]. The presence of the recessive *kti* gene in the BRS 155 allows a reduction in heat treatment, with consequent reduced processing costs and better protein solubility.

Crossing were performed in the summer of 2011 in a greenhouse and F_1 plants were obtained in the winter of 2012. Authentication of hybrid origin of F_1 plants was carried out and they were compared with their female parental. Freshly harvested seeds of these cultivars were analyzed for lipoxygenase content. Statistical analysis of obtained data was performed using the model for a completely randomized design with an unequal replication treatment (each family was considered a treatment). The genetic parameters were estimated using the Genes program [25].

2.2 Detection of Seed Lipoxygenases

To confirm the status of F_1 seeds and to check the segregation of $F_{2:3}$ seeds, non-destructive colorimetric analysis of LOX1 and LOX3 were performed by a procedure based on slightly modified colorimetric method of Suda et al. [26, 9] and single-dimension sodium dodecyl sulfate-polyacrylamide gel electrophoresis analysis (SDS–PAGE). Non-destructive analysis was achieved by cutting with a razor blade a small portion (5 to 10 mg) of cotyledons from the seed, in such a way that the rest of the seed would not lose its germination capacity. LOX1 was extracted by soaking 5 to 10 mg seed cotyledon in 0.25 ml water in a test tube for 3 min. After this period, 0.4 mL of a reaction mixture containing 130 mM sodium borate buffers, pH 9.5, 16 mM Methylene Blue, and 1.5 mM sodium linoleate was added to the tube. After 3 min the solution was checked for bleaching as a result of reduction of Methylene Blue (presence of LOX1) or not (absence of LOX1). LOX3 was extracted by soaking 5 to 10 mg seed cotyledon in 0.25 ml soybean extract containing only LOX1 (the test for LOX3 does not work properly in the absence of LOX1) for 3 min. After this period 0.4 ml reaction mixture containing 130 mM sodium phosphate buffers, pH 6.8, 4% (w/v) β-carotene dissolved in 50% (v/v) acetone, and 1.5 mM sodium linoleate. Bleaching of the β-carotene indicated the presence of LOX3.

Null $F_{2:3}$ families (based on the colorimetric assays) were extracted in lipoxygenase loading buffer [0.0625 mM Tris–HCl (pH 6.8), 2% SDS, 5% (v/v) beta-mercaptoethanol and 10% (v/v) glycerol] and separated by discontinuous (10% - 4%) SDS–PAGE [7]. Genotyping was performed by creating a binary array that was used for statistical analysis and construction of the genetic map.

2.3 Statistical Analysis

The individual segregation of seed lipoxygenases resulted from colorimetric analyses was tested using the

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chi-square test. Genetic distances between markers were estimated using the Mapmaker EXP V. 3.0 program [27]. A minimum logarithm of odds (LOD) score of 3.0 and maximum distance of 50 cM were chosen to establish the degree of linkage. Recombination fractions were converted to map distances using the Kosambi. LGs were named according to the designations of the consensus USDA map [28]. All analyses were carried out by GQMOL program [29].

2.4 Genotyping Assays

The DNA was extracted from the soybean leaves by the CTAB method [30], and then quantified in a spectrophotometer and stored at 4°C until use.

Out of more than 600 SSR markers developed by Cregan et al. [28], Song et al. [31] and Grant et al. [32] in soybean, 22 pairs of soybean primers flanking the microsatellite regions, previously published, were selected. They were synthesized by Bio Synthesis Inc., Texas, USA. Chromosome 15 (LG E): Satt575, Satt213, Satt651, Satt212, Satt598, Satt573, Sat_136, Satt606. Chromosome Gm13 (LG F): Sat_090, Satt656, Sat_417, Sat_074, Satt395. Chromosome 7 (LG M): Sat_389, Satt404, Sat_391, Satt636, Satt590, Satt201, Satt150, Sat_316, Satt567. The sequences of the Forward and Reverse primers are available at the soybean SoyBase, the USDA-ARS Soybean Genetics and Genomics Database [32]. The primers were chosen because they had presented polymorphism in previous studies [33, 20, 34, 13].

Markers that generated polymorphisms among the parents were amplified in DNA bulks of each family, for instance a solution containing equal amount of DNA from all the plants of the same family. The amplification reactions were carried out in a total volume of 15 µL, containing 10 mM Tris-HCl, pH 8.3; 50 mM KCl; 2.4 mM MgCl2; 100 µM of each desoxynucleotide; 0.3 µM of each primer, a unit of *Taq* polymerase and 30 ng genomic DNA. The amplification reactions were carried out in a Perkin Elmer thermocycler, model 9600, programmed for thirty cycles of 1 min at 94°C, 1 min at 50°C, and 2 min at 72°C; at the end of the 30 cycles, a stage of 7 min at 72°C was performed. The amplification products were separated by electrophoresis in 3% agarose gel, or in 10% native vertical polyacrylamide gels, using a TAE 1X buffer (0.09 M Tris- acetate and 0.002 M EDTA). The amplified SSR fragments of different sizes were considered as different alleles. The fragments were detected by silver staining, following Sanguineti et al. [35].

3. Results and Discussion

The phenotypes for LOX1 and LOX3 of the progenitors used in this work and of $F_{2:3}$ seeds derived from crosses among them were first confirmed by the colorimetric test, as shown in Figure 1. Blue color indicates absence of LOX1(-), yellow indicates presence of LOX1 (+), green indicates the absence of LOX1 (-) and LOX 3 (-); colorless indicates the presence of all lipoxygenases: LOX1 (+), LOX 3 (+).

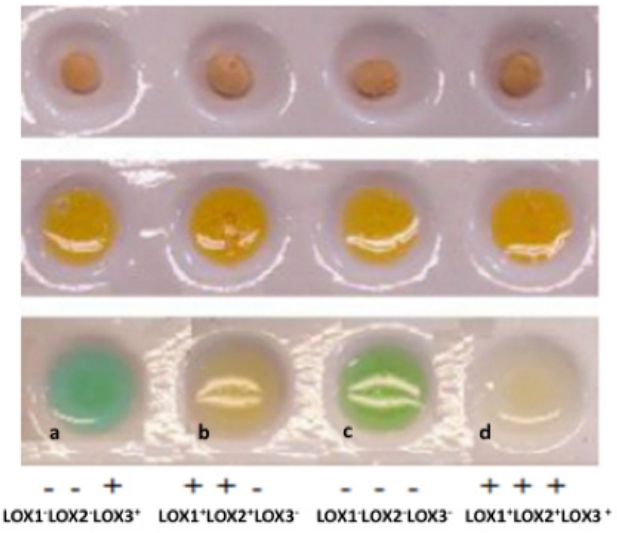

Figure 1. Colorimetric assay of soybean seed lipoxygenase enzymes for soybean in $F_{2:3}$ population from the cross between the soybean cultivars BR 36 and BRS 213, and between BRS155 and BRS213. a-blue indicates absence of LOX 1 (-); b-yellow indicates presence of LOX1 (+); c-green indicates the absence of LOX1 (-) and LOX 3(-); d-colorless indicates the presence of all lipoxygenase (+) LOX 1 (+), LOX 3 (+)

Results indicate a perfect association between the inheritance of homozygous lx_1 or lx_3 and the lack of LOX1 or LOX3 activity (Table 1).

The segregation of the $F_{2:3}$ RILs seeds derived from the cross between the triple null BRS 213 and BR 36 (dominant for the three lipoxygenase) a phenotypic ratio for LOX1 and LOX3 of 15:1 or 153:15 was observed and confirmed by the chi-square test at 1% significance (Table 1). This ratio is expected for two genes, which segregate independently.

The phenotypic segregation between BRS 213 and BRS 155 F2 RILs for the presence of the LOX1 enzymes carried out by the colorimetric test (Figure 1) allowed us to confirm the 3:1 segregation, 69 seeds showed presence of LOX1 and 24 seeds showed the absence of both enzymes; the chi-square test obtained was not significant at 1% probability (Table 1). The colorimetric assay indicated segregation to the presence and absence of lipoxygenase isozymes in the soybean seeds but did not allow detection

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Table 1. Colorimetric determination of presence or absence of LOX1 and LOX3 activity in $F_{2:3}$ soybean seeds population from the cross between the cultivars BR 36 and BRS 213 and between BRS155 and BRS 213.

Population $F_{2:3}$	Phenotype	Genotype	Expected [a]	Observed [b]	X^2	P
BR 36 X BRS213	Presence $LOX1^+LOX3^+$ And heterozygous $LOX1^+LOX3^-$	$Lx_1Lx_1Lx_3Lx_3$ and $Lx_1lx_1Lx_3lx_3$	157,5	153	$5,76^{ns}$	0,12
	Absence LOX1-LOX3-	$lx_1lx_1lx_3lx_3$	10,5	15		
BR 155 X BRS213	Presence LOX1+	Lx_1Lx_1 and Lx_1lx_1	70,0	69	$0,032^{ns}$	0,86
	Absence LOX1-	lx_1lx_1	23,5	24		

[a]Expected segregation ratio, [b]Observed segregation ratio; X^2 Chi-square value, [ns]Non significant at 1% probability; P: probability.

Table 2. Segregation analysis of three SSR markers (Satt212, Satt417and Sat_090) linked to the *Lx* genes in the $F_{2:3}$ population from the cross between the soybean cultivars BR 36 and BRS 213, and between BRS155 and BRS 213.

$F_{2:3}$ Population	LG	Marker	Genotype	Expected[a]	Observed [b]	X^2	P
BR36XBRS213	F	Satt212	lx_1lx_1,lx_3lx_3	38,75:77,50:38,75	27:79:43	$3,98^{ns}$	0,14
BRS155XBRS213	E	Satt417	lx_1lx_1	22,75:45,50:22,75	22:44:25	$0,29^{ns}$	0,86
		Sat_090	lx_1lx_1	23:46:23	23:46:23	$0,00^{ns}$	1,00

LG: Linkage group of linkage map of the soybean genome (Cregan et al., 1999); [a]Expected segregation ratio for co-dominant markers, X^2 Chi-square value, [ns]Non significant at 1% probability; P: probability.

of heterozygous individuals (Figure 1).

Although colorimetric analysis is useful during the breeding process to eliminate lipoxygenase from soybean seeds, it is very limited because no heterozygous seeds can be identified. Identification of heterozygous seeds can be extremely important in a backcross breeding program specially when the trait of interest is recessive. This information can speed up the creation of new cultivars because there is no need of self-fertilization during the odd numbered generations.

In the present study, we investigated two different seed-expressed lipoxygenases (Lx_1 and Lx_3), expressed in the Brazilian parental soybean cultivars BRS 213, BR 36 and in $F_{2:3}$ derived populations. Several authors reported the inheritance of seed lipoxygenases in soybean, showing that they are present on the syntenic region Ks0.1984 containing chromosomes 15 (E) and 13 (F). Lx_1 and Lx_2 genes are tightly linked and inherited together and the Lx_1 and Lx_2 genes are mapped as single major genes to the same location on chromosome Gm13 (LG F) while Lx_3 gene is independently inherited, mapped on chromosome Gm15 (LG E) [13]. Kim et al. [20, 34] found the Lx_2 locus positioned on one end of chromosome 13 (LG F), flanked by the SSR markers Satt522 and Sat074 and the Lx_3 locus at the linkage group M next to the marker Satt150. However, Reinprecht et al. [13] found Lx_2 locus flanked by the SSR markers Sat_090 and Sat_074 on chromosome 13 (LG F) and the loci Lx_3 was mapped on the chromosome 15 (linkage group E) next to the Satt212 [13, 14, 15]. Thus, in order to validate the SSR markers reported previously by Kim et al. [33], Kim et al. [20], Reinprecht et al. [13], Lenis et al., [14] and Reinprecht et al. [15], we surveyed the DNA

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polymorphism in the parents cultivars and RIL $F_{2:3}$ derived population generated from the cross between BRS 213 ($lx_1lx_2lx_3$) and BR36 ($Lx_1Lx_2Lx_3$) and BRS 213 ($lx_1lx_2lx_3$) and BRS 155 ($Lx_1Lx_2Lx_3$). Additional SSR markers in the neighboring genomic region of Lx_1 and Lx_3 were tested.

Among the 22 SSR primer pairs used in PCR amplification of DNA for the parents lines BRS 213, BR 36 and BRS155, only eight produced polymorphic DNA fragments showing 31% of parental polymorphism. Nevertheless, seven SSR markers which have shown polymorphisms in the parents cultivars had shown poor segregation in the $F_{2:3}$ population and the data were excluded. The SSR primers pair previously used by Kim et al. [33] linked with Lx_1 (Lx_2) have shown polymorphism in the parental lines BRS 213 and BR 36 but did not in the RIL $F_{2:3}$ derived population. However, the RIL $F_{2:3}$ population derived from cross BRS155 and BRS 213 presented polymorphism to the primers linked to Lx_1,(Lx_2) locus but did not reveal polymorphism to the marker linked to lx_3 locus.

Therefore, the RIL population $F_{2:3}$ derived from the cross between BR36 and BRS213 were used to map locus Lx_3 and the RIL population $F_{2:3}$ derived from cross between BRS155 and BRS213 were used to map the locus Lx_1(Lx_2).

The poor polymorphism observed in the current study appeared to be quite similar to the one found by other authors [36, 13, 14, 15, 21, 37]. This approach was explained previously based on the fact that the commercial soybean cultivars fell into 17 allelic classes from five genotypes and the pedigree of these genotypes are closely related. Moreover, the use of markers in breeding depends on their being polymorphic, because there is no warranty that the markers identified in one population are polymorphic in different populations.

The level of polymorphism in soybean depends on the genetic background of the parents used to develop the populations, as well as on the type of marker used in a study [36]. Low polymorphism observed in this study could be explained by the fact that all three populations share the similar ancestral, all of them are descended either from Century L1L2 as source of null lipoxygenase [38, 18] or from a selection of this cultivar.

Lenis et al. [14] developed gene specific molecular markers assay for genotyping of three triple null soybean lipoxygenase free breeding lines IA2040LF, 8AR-56061 and 935F203. They concluded that there were no new mutations in the US lines, but the lipoxygenase genes took part in the triple null quality of the ancestral triple null lipoxygenase Jinpumkong 2 [20,33] that the new soybean lines are derived and the genetic recombination was the mechanism that broke the tight repulsion-phase linkage between Lx_1 and Lx_2 loci, allowing the combination of three independent *lx* mutant alleles in the lipoxygenase-free.

The PCR product of parental cultivars BRS 213 and BRS 36 showed polymorphism to the SSR marker Satt212. The SSR marker Satt212 appeared linked and codominant segregation for the 149 RILs (Figure 2). The populations showed normal segregation of 1:2:1 ratio with a Chi-squared value of 3,98 (P = 13,67) (Table 1). It was also observed that the Lx_3 locus segregated independently of the Lx_1 loci. A genetic map was constructed using genotyping data of Satt212 and phenotyping data for Lx_3 locus (Figure 3). The Lx_3 locus showed complete association between the inheritance of homozygous *lx* mutant alleles and the lack of lipoxygenase activity. Lx_3 free has been located on chromosome Gm15 linkage group E in the classical linkage map of soybean [28]. The distance of Satt212 from Lx_3 gene was 24,1 cM, corresponding with the distance assigned by Reinprecht et al. [13]. Though it is the first report of a map position for LOX3 free in Brazilian commercial cultivar. However, the discovery of still closer SSR marker will make the assisted selection for this gene more accurate.

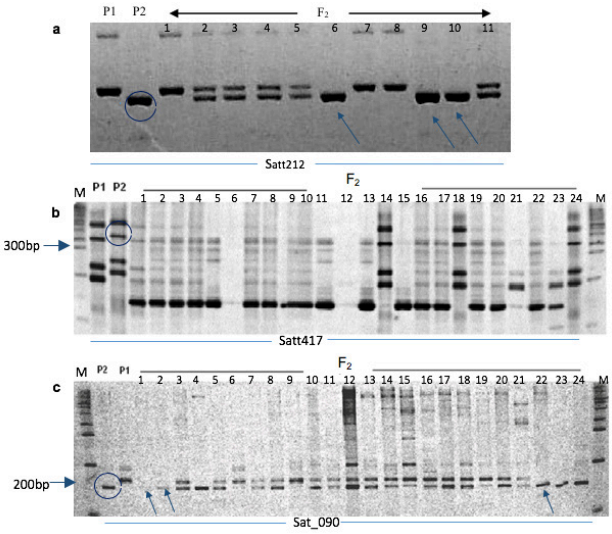

Figure 2. Amplification products of three SSR markers linked to the soybean lipoxygenase gene. M is a 100 bp DNA ladder. a. Segregation pattern of DNA fragments in parents P_1 BR 36 and P_2 BRS 213 $F_{2:3}$ RIL population using SSR markers Satt212, lanes 6, 9 and 10 absence of LOX3; b. Segregation pattern of DNA fragments in parents P_1 BRS 155 and P_2 BRS 213 and F_2 RIL using SSR markers Satt 417, all lines shows presence of LOX1; c. Segregation pattern of DNA fragments in parents P_1 BRS 155 and P_2 BRS 213 and $F_{2:3}$ RIL using SSR markers Sat_090, lines 1, 2, 4 and 22 shows absence of LOX1. The arrow depicts band fragment size of lipoxygenase triple null mutant from BRS 213

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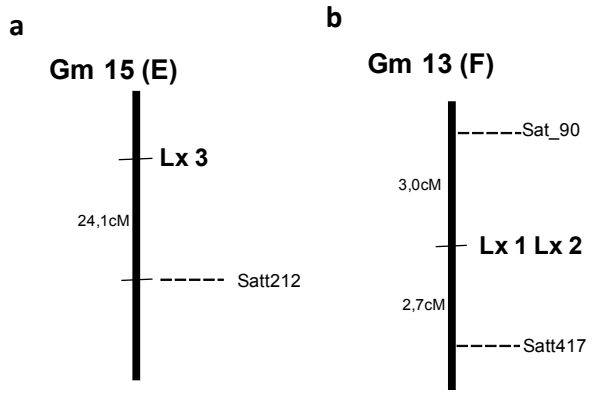

Figure 3. a. Position of the seed lipoxygenase Lx_3 gene mutation-based markers in the $F_{2:3}$ population BRS 213(lx_1, lx_2; lx_3) X BR 36 ($Lx_1Lx_2Lx_3$). Lx_3 is mapped on chromosome Gm15 or linkage group E. b. Position of the seed lipoxygenase Lx_1 and Lx_2 gene mutation-based markers in the $F_{2:3}$ population BRS 213(lx_1, lx_2; lx_3) X BRS 155 ($Lx_1Lx_2Lx_3$). Lx_1 and Lx_2 are mapped on chromosome Gm13 or linkage group F. The linkage map was aligned with the soybean composite_2003 map (www.soybase.org) and linkage map of the soybean genome (Cregan et al., 1999). Name of the locus is indicated on the right side of each chromosome. Marker is connected by dashed line

The markers for the mutation in the Lx_1 gene and those previously reported in the vicinity, Sat_074, Sat_090, Satt395, Sat417, Satt656, were tested for parental polymorphism in the lines BRS 213 and BRS 155 and were found to be polymorphic to the parental lines. The others markers tested did not reveal polymorphism on the parental cultivars. Genotyping of Lx_1 against the 93 RILs was successfully conducted using the SSR markers Sat_090 and Satt417. Sat_090 Showed normal segregation of 1:2:1 ratio with Chi-squared of 0,00 (P = 100%) and Satt417 showed a normal segregation of 1:2:1 ratio with a Chi-squared of 0,297 (P = 86,21%) (Table 1). Representative gel of amplification pattern obtained for Sat_090 and Satt417 using the genomic DNA of the parents BRS 213 and BRS 155 and the $F_{2:3}$ population is shown in Figure 2.

The Lx_1 gene for LOX1 has been mapped to chromosome 13 LG F in the molecular soybean genetic map [28]. A genetic map was constructed using genotyping data of Sat090 and Satt 417. Lx_1 loci has been located on linkage group F in the classical linkage map of soybean [28]. This indicates that Lx_1 is located nearly at the same position as Lx_2 in our study (Figure 3). Sat090 was found to be linked with Lx_1 and Lx_2 loci at a distance of 3,00 cM and 2,77 cM respectively, the two SSR markers are flaking the Lx_1, Lx_2 in interval distance of 5,77 cM (Figure 3). This is in accordance with the observation of Kim et al. [20] and Reinprecht et al. [13, 15].

Satt090 and Satt417, on the same linkage group, presented high efficiency in MAS because they are very close to Lx_1, Lx_2 genes. In addition, the fact that the two markers are flanking the genes increases selection efficiency. Phenotypic selection is influenced by the environment and the number of plants that can be selected is limited, while assisted selection by molecular markers does not have such limitations. Therefore, the SSR markers validated in this study will allow more accurate selection for seed lipoxygenase nulls on base of DNA screens rather than relying on phenotypic expression with the potential to simplify and accelerate selection for further application in the Brazilian breeding program aimed at improving soybean seed quality.

4. Conclusions

The absence of lipoxygenase is due to the action of three recessive genes (Lx_1, Lx_2 and Lx_3) and in the RILs population between BR 36 and BRS 213 and between BRS 155 and BRS 213 the nulls genes come from BRS 213.

The results confirm the linkage of SSR marker Satt212, linkage group E, with the locus Lx_3 in the RILs population derived from BR 36 and BRS 213, at distance of 24.1 cM.

The RILs population derived from BR 36 and BRS 213 did not present polymorphism to the SSR markers of LGF, thus it was not possible mapping Lx_1 and Lx_2 loci.

For the RILs population derived from BRS 213 and BRS 215, the SSR markers Satt090 and Satt417, linkage group F, were found to be linked with the Lx_1, and Lx_2 at a distance of 3,00 cM and 2,77 cM, respectively.

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ARTICLE

Identification, Structure Analyses and Expression Pattern of the ERF Transcription Factor Family in *Coffea arabica*

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ABSTRACT

Members of the ERF Family of Transcription Factors play an important role in plant development and gene expression that regulates responses to biotic and abiotic stress. This work identified 36 ERF family genes in *Coffea arabica* within the AP2/ERF full domain, using the EST-based genomic resource of the Brazilian Coffee Genome Project. The ERF family genes were classified into nine of the ten existing groups through phylogenetic analysis of the deduced amino acid sequences and comparison with the sequences of the ERF family genes in Arabidopsis. In addition to the AP2 domain, other conserved domains were identified, typical of members of each group. The *in silico* analysis and expression profiling showed high levels of expression for libraries derived from tissues of fruits, leaves and flowers as well as for libraries subjected to water stress. These results suggest the participation of the ERF family genes of *C. arabica* in distinct biological functions, such as control of development, maturation, and responses to water stress. The results of this work imply in the selection of promising genes for further functional characterizations that will provide a better understanding of the complex regulatory networks related to plant development and responses to stress, opening up opportunities for coffee breeding programs.

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1. Introduction

With an annual worldwide production of 168.5 million of 60 kilograms bags of grains in 2019 [1], coffee is an important agricultural commodity cultivated in more than 80 countries, which represents a significant source of income mainly for developing tropical countries[2]. Brazil is the largest world producer and, together with Vietnam and Colombia, accounts for more than 50% of the world production [1]. Among the 124 identified species, only two are economically important: *Coffea arabica* and *Coffea canephora* [3]. The world market shares for these two species are 70% and 30%, respectively.

During their life cycle, crops are exposed to various biotic and abiotic stresses that limit their growth, development, and production [4,5]. To survive in stress conditions, plants have developed a complex molecular signaling network [6,7]. Gene regulation by transcription is one of the main control points of biological processes in which Transcription Factors (TFs) play a central role [8,9].

AP2/ERF superfamily is composed of ERF (Ethylene Responsive Factor), AP2, and RAV families, which consists of about 60-70 amino acids involved in DNA binding [10]. The ERF family proteins contain a single AP2 domain and the AP2 family proteins contain two repeated AP2 domains [11]. In addition to the single AP2 domain, the RAV family proteins contain a B3 domain that is a DNA-binding domain [12, 13]. The ERF family is further divided into two subfamilies: the ERF and the CBF/DREB [11, 13, 14].

Generally, the ERF family genes are partially involved in responses to biotic stress by recognizing the cis-acting sequence AGCCGCC, known as GCC-*box* [15]. The CBF/DREB subfamily genes play a crucial role on the plant responses to abiotic stress by recognizing the dehydration responsive element (DRE) with a central motif A/GTCGAC[16, 17]. The roles of the ERF and CBF/DREB proteins on the development and response to biotic and abiotic stresses in different plant species have been widely studied. Combining molecular genetic approaches, a series of ERF family regulatory genes involved in different metabolic pathways have been examined, including those related to drought [5, 18, 19, 20, 21], salinity [18, 22], low temperatures [23, 24, 25], and diseases [26, 27]. In addition to responses to diverse stress types, the ERF family genes are also involved in the development of roots [28], germination [29, 30], and development and maturation of fruits [31, 32].

Transcription Factors of the ERF subfamily and the CBF/DREB subfamily were identified in diverse species: Arabidopsis [14, 13, 33], rice [13, 34], cotton [35, 36], soybean [37], poplar [38], grape [15, 39], corn [40], tomato [8], apple [41], citrus [42] and banana [7]. Few studies with ERF family members in *Coffea* ssp were published [43]. Bustamante-Porras et al. [44] isolated the first ERF family member in *C. canephora*, whose expression is involved in processes of cell differentiation and fruit maturation. In *C. arabica*, no member of the ERF family was described until this moment.

Research on genomics and transcriptomics in coffee has gained more prominence. The Brazilian Coffee Genome Project [45, 46] has been developed to investigate the coffee characteristics through complementary DNA sequencing (cDNA). This database has a set of 265,889 expressed sequence tags (ESTs) from different tissues for *C. arabica, C. canephora* and *C. racemosa*. Therefore, the aim of this work was to identify and characterize possible ERF transcription factors in *C. arabica* from the ESTs database of the Brazilian Coffee Genome Project.

2. Material and Methods

2.1 Identification and Classification of ERF Family Genes in *Coffea arabica*

The ERF family genes in *C. arabica,* searches on the Brazilian Coffee Genome Database (http://bioinfo03.ibi.unicamp.br/cafe/) were performed using the AP2 domain of the *Solanum lycopersicon* ERF4 protein (GENBANK: AAO34706), with the BlastP software [47]. More than 265,889 ESTs sequences are available in this database, which were obtained from forty-three cDNA libraries, most of them of *C. arabica.* The cDNA was obtained from different tissues of the coffee plant (leaves, roots, flowers, seeds, fruits, among others) in different stages of development and subjected to various stress conditions [45, 46]. In order to increase the chances to identify new ESTs, searches were also performed using the following keywords: ERF, Ethylene Response Factor and EREBP. To verify the specificity of the annotated sequences, comparisons were confronted using BlastP tool with other sequences deposited in the NCBI database (http://www.ncbi.nlm.nih.gov/protein/). The deduced sequence of amino acids of each *contig* was obtained by the ORF Finder *software* (Open Reading Frame Finder - NCBI -https://www.ncbi.nlm.nih.gov/orffinder/). The sequences that presented incomplete AP2 domain or incorrect ORFs were excluded from analysis.

2.2 Phylogenetic Analysis

The protein sequences of the AP2 domain were aligned by the Clustal Omega algorithm version 2.0.3 [48]. The phylogenetic tree was constructed using the the MEGA *software* version 7.0 [49] based on neighbor-joining (NJ) method with *pair-wise* deletion, and the reliability was tested with 1,000 iterations of the *bootstrap.*

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Table 1. *Coffea arabica* sequences with identity to ERF gene family in *Arabidopsis thaliana*

Arabidopsis thaliana		*Coffea arabica*			
Gene	Gene	ERF	Coverage (%)	e-value	Identity (%)
AT1G78080	RAP2.4	CaERF01	99	1,00E-68	81
AT1G78080	RAP2.4	CaERF02	97	2,00E-63	81
AT1G78080	RAP2.4	CaERF03	100	2,00E-58	80
AT1G78080	RAP2.4	CaERF04	100	5,00E-62	80
AT1G78080	RAP2.4	CaERF05	100	2,00E-34	81
AT5G67190	AtERF10	CaERF06	100	3,00E-39	74
AT5G52020	AtERF25	CaERF07	63	8,00E-30	71
AT5G52020	AtERF25	CaERF08	85	6,00E-45	65
AT244940	AtERF34	CaERF09	65	6,00E-48	77
AT240340	DREB2C	CaERF10	84	9,00E-49	68
AT240340	DREB2C	CaERF11	78	8,00E-38	70
AT1G75490	DREB2D	CaERF12	87	3,00E-37	69
AT4G27950	CRF4	CaERF13	71	3,00E-30	63
AT3G16770	ATEBP/RAP2.3	CaERF14	96	8,00E-34	72
AT3G16770	ATEBP/RAP2.3	CaERF15	93	2,00E-29	73
AT3G16770	ATEBP/RAP2.3	CaERF16	96	2,00E-29	68
AT3G16770	ATEBP/RAP2.3	CaERF17	62	1,00E-28	81
AT3G16770	ATEBP/RAP2.3	CaERF18	90	6,00E-27	81
AT3G14230	RAP2.2	CaERF19	99	8,00E-33	77
AT3G16770	ATEBP/RAP2.3	CaERF20	85	8,00E-28	77
AT3G16770	ATEBP/RAP2.3	CaERF21	96	1,00E-27	84
AT3G15210	AtERF4/RAP2.5	CaERF22	42	3,00E-30	74
AT3G15210	AtERF4/RAP2.5	CaERF23	41	5,00E-30	74
AT1G50640	AtERF3	CaERF24	82	2,00E-42	76
AT5G44210	ATERF9	CaERF25	97	4,00E-30	76
AT1G28360	ATERF12	CaERF26	68	1,00E-26	85
AT4G17500	AtERF1	CaERF27	75	9,00E-53	75
AT4G17500	AtERF1	CaERF28	86	1,00E-44	73
AT4G17500	AtERF1	CaERF29	79	5,00E-52	74
AT3G23240	ERF1	CaERF30	82	4,00E-31	73
AT4G17490	AtERF-6	CaERF31	97	5,00E-42	77
AT4G17490	AtERF-6	CaERF32	84	4,00E-31	78
AT4G17490	AtERF-6	CaERF33	93	7,00E-34	74
AT3G23240	ERF1	CaERF34	54	2,00E-15	73
AT5G61890	ABR1	CaERF35	94	2,00E-33	90
At2G33710	AtERF112	CaERF36	49	4,00E-28	75

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2.3 Determination of Conserved Motifs

The identification of conserved motifs in protein sequences of *C. arabica* was analyzed using the Meme Suite version 4.11.2 (http://meme-suite.org/tools/meme), with the following parameters: ideal size: 6-80 amino acids; any number of repetitions for motifs and maximum number of motifs = 25. The resulting motifs were verified in the databases of NCBI (http://www.ncbi.nlm.nih.gov/gorf/gorf.html) and PROSITE (http: //www.expasy.org) to verify their significance.

2.4 Gene Expression Profiling by Electronic Northern Blot

The gene expression profiles were performed by the Northern Blot technique. The specific tissue libraries investigated in this study were from Brazilian Coffee Genome Database [45]. The frequency of *reads* in each library was normalized according to Lima et al. [43]. The e-Northern Blot was performed using Genesis *software,* version 1.7.5.

3. Results

3.1 Identification and Phylogenetic Relationships of ERF Family in *C. arabica*

The analysis comprised a data mining process within the Coffee Genome Project Database to identify the ERF family members in *C. arabica*. For this, the *reads* that possibly encode the AP2 domain in *C. arabica* were selected. The search by keywords and through the AP2 domain of ESTs enabled the identification of 38 Transcription Factors encoding ERF proteins. Among these 38 possible ERFs, it was observed that only 36 ERF proteins contained a full AP2 domain, while the other 2 ERFs contained only a part of the AP2 domain and, therefore, were excluded from analysis. The identity of the ERF proteins in *C. arabica*, with their homologs in Arabidopsis, varied from 63-90% (Table 1). The conservation of the sequence in comparison with arabidopsis was higher in the Group X members, varying from 75-90%. Lower values were observed in the Groups III (65%) and VI (63%).

A phylogenetic reconstruction was obtained from the identification of 122 ERFs in Arabidopsis, previously described by Sakuma et al. [14]. The ERF sequences are highly conserved between species. It favored the distinction of the 10 main groups, named as Groups I-X by Nakano et al. [13]. According to Figure 1, the comparative analysis of the phylogenetic tree for Arabidopsis and *C. arabica* grouped a high number of identified sequences in *C. arabica* (22.22%, 8 sequences of 36) together with the arabidopsis sequences belonging to the Groups VII and IX. In *C. arabica,* a lower number of proteins was grouped into the ERFs of the Arabidopsis belonging to the Groups I and VIII (13.89%, 5 sequences), followed by the Groups III and IV (8.33%, 3 sequences). The ERF groups with fewer members in *C. arabica* were X, II and VI (5.56%, 2.78% and 2.78%; related to the sequences 2, 1 and 1, respectively). No member of *C. arabica* was found in the Group V and in the Sub-Groups VI-L and Xb-L. These two subgroups are characterized in Arabidopsis by a low homology in the C-terminal region of the AP2/ERF domain [36].

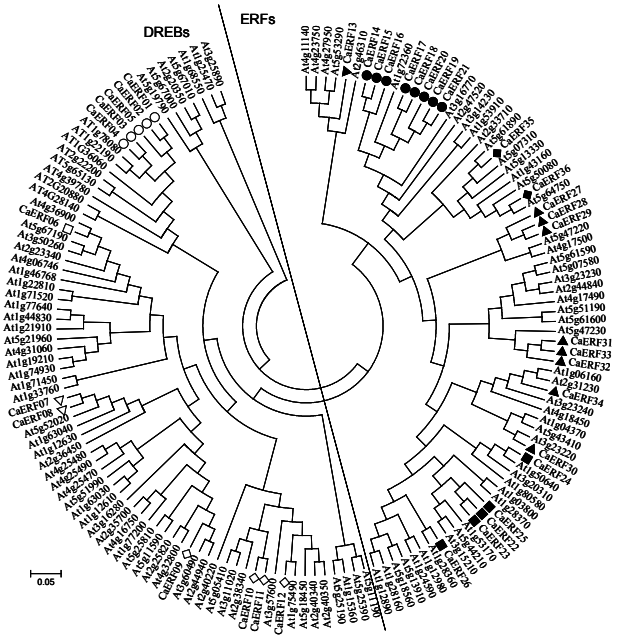

Figure 1. Phylogenetic tree of the ERFs in *C. arabica* and Arabidopsis . The phylogenetic tree was constructed with MEGA 7.0 using the NJ method. (DREB subfamily: ○ - Group I, □ - Group II, ▽ - Group III, ◇ - Group IV; ERF subfamily: ▼- Group VI, ● – Group VII, ■ - Group VIII, ▲ – Group IX, ◆ - Group X).

The ERFs were divided into two subfamilies. It was identified 12 putative ERFs as members of the DREB subfamily (Groups I, II, III and IV), in comparison with 57, 40, 75 and 58 in Arabidopsis, grape, poplar and rice, respectively. It was identified 24 encoding genes within the ERF subfamily (Groups VI, VII, VIII, IX, X) in comparison with 65, 82, 134 and 87 in Arabidopsis, grape, poplar and rice, respectively. The organization of the ERF family genes in *C. arabica* is showed in Table 2 along with the comparative distribution of Arabidopsis, grape, poplar and rice.

To study the phylogenetic relationship between the ERF family genes of *C. arabica* and Arabidopsis*,* multiple analyses were realized with the alignment of the deduced sequences of amino acids. The alignment of the AP2 do-

Table 2. Number of genes in each Group of the ERF Family in *C. arabica* and in species whose genome was completely sequenced and size of the respective genomes.

Family Subfamily	Group	Arabidopsis[a]	Vitis[b]	Poplar[c]	Rice[a]	Coffe[a]
ERF	I	10	5	8	9	5
	II	15	8	51	16	1
DREB	III	23	22	6	27	3
	IV	9	5	10	6	3
	V	5	11	42	8	0
	VI	8	2	20	6	1
	VI-L	4	5	-	10	0
ERF	VII	5	3	12	15	8
	VIII	15	11	39	15	5
	IX	17	40	19	18	8
	X	8	10	2	12	2
	Xb-L	3	0	-	3	0
Total ERFs		122	122	209	145	36
Genome Size (Mb)		125	487	465	430	1.300

[a]Nakano et al. [13], [b]Licausi et al. [15] and [c]Wang et al. [38].

main indicated that the residues Gly-4, Arg-6, Glu-16, Trp-28 and Gly-30 are completely conserved among all proteins within the ERF family in *C. arabica* and Arabidopsis. Furthermore, more than 95% of the ERF family members contain the conserved residues Arg-8, Gly-11, Ile-17, Arg-18, Arg-26, Leu-29, Ala-38, Ala-39, Asp-43 and Asn-56. As previously demonstrated by Sakuma et al. [14] the ERF gene subfamily includes two main residues of amino acids, the alanine (A) at position 14 and the aspartate (D) at position 19, which possibly contribute to a functional activity of binding to GCC-box in many ERFs. The CBF/DREB family contains a valine (V) and a glutamic acid (E) at positions 14 and 19, respectively. In the DREB subfamily, all genes present the conserved residue Valine14 and, at position 19, the genes *CaERF01-05* contain 1 Leucine (L) and the genes *CaERF06-12* contain 1 Glutamic Acid (E). The C-terminal region of the AP2/ERF domain of the *CaERF34* protein showed low homology with the region of consensus with other genes (Figure 2). This region corresponds to the half of terminal *α-helix* [50], which includes the highly conserved residues Asp-43 and Asn-57. In general, the ERF family showed significant similarity to the remaining domain.

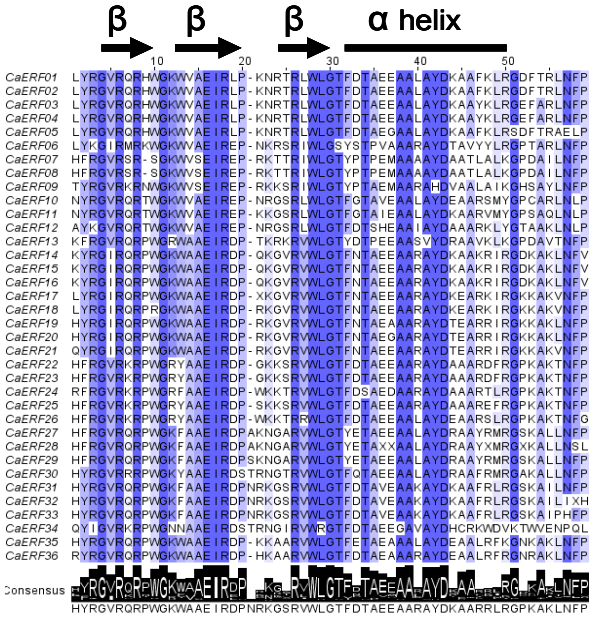

Figure 2 – Multiple sequence alignment of *C. arabica* AP2 domains from the ERF proteins. Identical and conserved amino acid residues are represented by dark and light blue shading, respectively. Black bar and arrows represent the predicted *α-helix* and *β-sheets* regions, respectively. *CaERF*01-12 belongs to the DREB subfamily; *CaERF*13-36 belongs to the ERF subfamily.

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3.2 Distribution of Conserved Motifs

In general, the regions in Transcription Factors outside the DNA-binding domain contain important domains that are involved in transcription activities as the protein-protein interactions, which may be involved in nuclear localization [33]. Such functional domains are often conserved among members of a subgroup within families of transcription factors in plants. Probably these motifs are sharing the same functions [13, 51, 52].

In order to relate the putative ERFs in *C. arabica* to biological functions, other conserved motifs (CM) outside the AP2/ERF region were investigated on the deduced sequences of amino acids. Most members of the same group shared one or more motifs outside the AP2 domain (Figure 3). For instance, the Group I comprise 5 ERFs (*CaERF*01-05) and contain 5 conserved motifs (Figure 3). Except *CaERF*05, the members of this group contain the CMI-1 and CMI-2 motifs in the C-terminal region. The *CaERF*06 gene belonging to the Group II, unique member identified in this work, contains the CMII-1motif in the C-terminal region, adjacent to the AP2 domain. Belonging to the Group III are the *CaERF*07-09 genes, which contain the CMIII-1 motif in the C-terminal region. In addition to the CMIII-1 motif, the *CaERF*08 gene contains CMIII-2 and CMIII-4, and the last is identified as the LWSY conserved motif in the *OsDREB1A/B/C* and *AtCBF3/DREB1A* [18]. The *CaERF*09 represents the CMIII-6 and the CMIII-7 motifs. In the Group IV (*CaERF*10-12) only the CMIV-2 motif was found in the *CaERF*10 and *CaERF*11

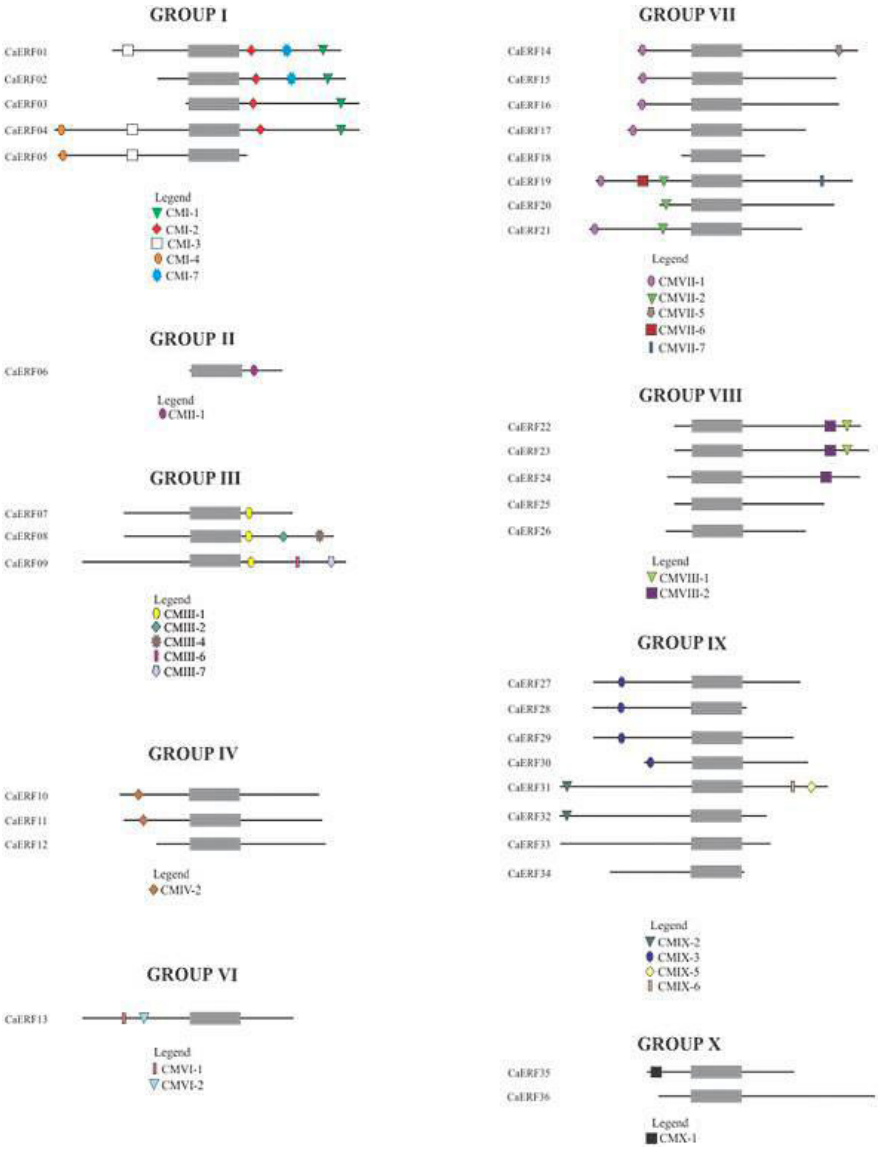

Figure 3. Conserved motifs in the ERF family in *Coffea arabica.* The motifs were identified in *C. arabica* and classified according to the classification proposed by Nakano et al. [13].

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genes. The CMIV-2 motif includes a putative nuclear localization signal [33].

The *CaERF*13 gene, unique of the Group VI, has two proteins that share the CMVI-1 and CMVI-2 conserved motif on the N-terminal region. The Group VII was firstly described by Tournier et al. [53] and is characterized by the presence of one highly conserved motif in the N-terminal region (MCGGAII/L). Within this group, 8 members were found in *C. arabica*. The EAR motif (CMVIII-1) was found in members of the Group VIII, in the ERFs *CaERF*22 and *CaERF*23, which also contain the CMVIII-2 motif. The Group IX is composed by 8 genes (*CaERF*27-34). The *CaERF*27-30 genes contain only the CMIX-3 motif while the *CaERF*32 gene contains only the CMIX-2 motif. The CMIX-3 motif corresponds to a conserved sequence that was referred previously to a DMLV motif [26]. In addition to the CMIX-3, the *CaERF*32 gene contains the CMIX-5 and CMIX-6 motifs that are probably a MAP kinase phosphorylation site, located at the C-terminal region of the protein [54]. The Group X is represented by the *CaERF*35 and *CaERF*36 genes. The group X members contain one CMX-1 conserved motif in the N-terminal region, such as the *CaERF*35, whereas the *CaERF*36 presents no conserved motif.

3.3 In Silico Gene Expression Profiling of the ERF Family in *Coffea arabica*

In order to assess the differences among transcripts of different tissues or organs, the ERF expression profiling was obtained in silico by e-Northen in the *C. arabica* libraries. High levels of expression were observed in libraries derived from tissues of fruits, leaves and flowers (Figure 4). The ERFs of the cDNA libraries from the tissues subjected to different types of stresses were also detected, however, with fewer transcripts than those from the tissues of diverse parts of the plant and different stages of development. Transcripts were detected in the majority of the evaluated libraries for the *ERFs CaERF20, CaERF21* and CaERF23. However, the majority of the 36 transcription factors of the ERF family were detected in specific libraries, showing that they are tissue specific. This is the case of *CaERF03, CaERF08, CaERF12, CaERF18, CaERF22, CaERF26, CaERF31* and *CaERF36,* which are expressed only in fruits. On the other hand, the *CaERF03, CaERF08, CaERF12, CaERF18, CaERF22, CaERF26, CaERF31* and CaERF36 genes are expressed only in flowers, leaves and roots. Expression profiling in libraries subjected to water stress were observed for *CaERF06, CaERF07, CaERF11, CaERF21* and *CaERF23*; the first three ERFs showed a higher expression in this library.

4. Discussion

Transcription factors are the principal regulators of biological factors and emerged as a powerful tool to manipulate complex metabolic pathways [55,56]. Using these proteins on plant breeding programs involves knowledge

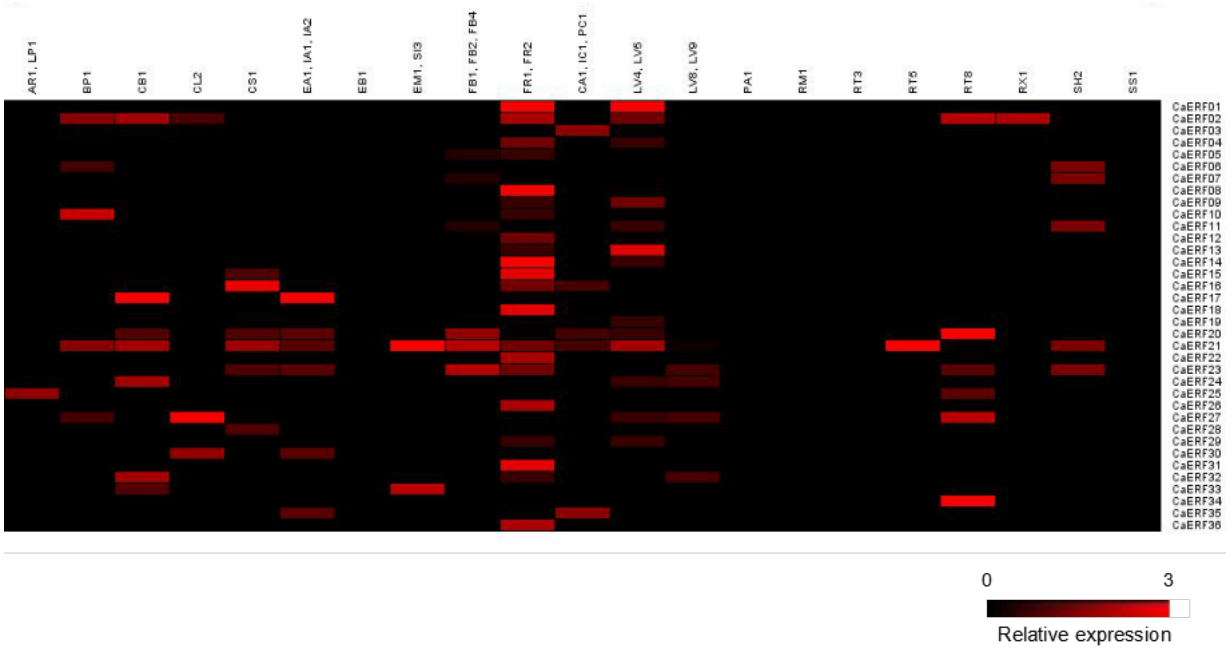

Figure 4. In-silico expression profiling of the ERFs in *Coffea arabica*. The number of reads was normalized in each library and values were represented by the relative expression scale.

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on their role in gene regulatory networks. The ERF family of transcription factors presents a highly conserved element including the AP2/ERF domain, responsible for the DNA binding activity and important to plant development[14, 57, 58]. Nakano et al. [13] have systematically studied the phylogeny, the structures, and the conserved motifs of the ERF family in Arabidopsis and rice. In order to obtain more information on this family in *C. arabica*, this present work identified and analyzed 36 possible ERF proteins from the EST database in *C. arabica*, which is available at the Brazilian Coffee Genome Project [45, 46].

The AP2 conserved domains of Arabidopsis were used to group their homologs in *C. arabica*. The majority of the sequences in *C. arabica* are grouped into Groups VII and IX followed by the Groups I and VIII. Only few sequences were grouped into Groups III, VI, X, II and VI. However, the Group VII represents about 4.1% of the family in Arabidopsis [13], 2.46% in grape[15], 9.56% in poplar[38] and 10.34% in rice [13]. This group represents more than 22% of the proteins containing the single AP2 domain, found in the Coffee Genome database. In this work, from the ten main groups identified in the ERF family in Arabidopsis, nine occurred in *C. arabica*. Therefore, the methodology used by Nakano et al. [13] is applicable in this species. The presence of the majority of groups and subgroups in the two dicot species, as well as in monocot species suggests that many of the genes predate the divergence between monocotyledonous and dicotyledonous [8]. On the same way, some groups and subgroups are present in only one specie, for instance, the Groups XI-XIV occur only in the ERF family in rice, excluding Arabidopsis and other dicot species. It suggests that these groups have evolved or were lost in a certain species after divergence [37].

The structural analysis revealed that all EFR proteins contain conserved Ala-14 and Asp-19, whereas the DREB proteins contain Val-14 and Glu/Leu-19. The comparative analysis of the amino acids residues of the ERF/AP2 domain in *C. arabica* with the ERF family proteins in *Arabidopsis* showed that the AP2/ERF domains were well conserved between the two species. These conserved amino acids probably play an important role in the ERF gene family, where they can be involved with different ways of contact with DNA. According to Allen et al. [50] the AP2/ERF domain recognizes the DNA by the conserved residues arginine and tryptophan, located into *β-sheets*. The Ala-37 in the ERF domain plays an important role in the stability of the ERF domain or DNA binding to the DRE element or GCC-*box* [59].

The transcription factors generally contain conserved domains that are outside the DNA binding domain, but functionally important [60,61]. The distribution of the specific motifs into proteins belonging to the specific groups of the phylogenic tree was also observed for the ERF proteins in *C. arabica*, which demonstrated structural similarities within the same subgroup. The majority of the ERF sequences identified in *C. arabica* share one or more motifs outside the AP2 domain with their homologs in Arabidopsis, such as in rice and soybean [13, 37]. For instance, Ohta et al. [57] identified an EAR motif (ERF related to amphiphilic repression), which works as a repression domain. The EAR motif of conserved sequence, (L/F) DLN(L/F)xP, identified in this present work as CMVIII-1, is found in the C-terminal regions of the Group VIII. This motif was already identified in various repressors, including ZAT7, 10, 12, ERF3, AUX/IAA, NIMIN1, HSI2, SUPERMAN (Arabidopsis), NRR (rice), ZFT1 (tobacco) and ZPT2-3 (petunia), which play different roles - from the plant development to stress tolerance[62,63,64]. Currently the DEAR1, a DREB protein containing the EAR motif, appeared as a protein repressor of binding to dehydration responsive element, which mediates responses to biotic and abiotic stresses[65]. The CMIV-2 motif in the N-terminal region could work as a nuclear localization signal (NLS) [66]. Recently, it has been considered essential in Arabidopsis that CBF1 bind to DNA, since it is indispensable for transcriptional activity [67]. A putative nuclear localization signal (KRKRK) was identified in ERF proteins [31, 68]. The comparative analysis of the conserved motifs in *C. arabica* and Arabidopsis suggests that the protein functions were conserved and diverged during the evolution of the ERF gene family. Sharma et al. [8] showed that some motifs are specific in spermatophytes whereas many motifs have been identified in non-vascular plants, bacteria, fungi and animals. The presence of these conserved motifs in evolutionarily different organisms indicates that they play an important functional role, while specific motifs in spermatophytes may have later evolved to fulfill specific functions.

In this present work, 8 ERFs belonging to the Groups I, III, VI, VII, VIII, IX and X were expressed only in fruit libraries. Although the ERF transcription factors are regulated by a series of physical and chemical stimuli, many ERFs are responsive to ethylene, and therefore they may be involved in the maturation process of climacteric fruits. Tournier et al. [53] demonstrated that the *SlERF2*, an ERF that binds to the GCC-*box*, plays an important role during the tomato maturation process. The same was observed by Yin et al. [69] for different ERFs expressed during the kiwi maturation process. Pereira et al. [70] showed that the autocatalytic production of ethylene in fruits of green *C. arabica* is very low; however, it increases considerably during the initial stage of ripening. Such observations

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demonstrate the climacteric nature of the maturation of *C. arabica* fruits and the importance of ethylene in this process. Bustamante-Porras et al. [44] isolated an ERF gene (*CoERF*) in *C. canephora*, with expression during fruit differentiation and maturation. Comparing this *CoERF* (GENBANK: AY522505) with the *CaERF*17 in *C. arabica*, it shared 97% of identity and 98% of similarity. Given that *C. canephora* is one of the ancestors of *C. arabica*, the *CaERF*17 was expected to be expressed in fruits. However, *reads* were not found in fruits libraries for *CaERF*17. In allotetraploids, genes are expected to be present in two homologous forms, highly similar, but not identical [71]. The redundancy of genes can lead to gene silencing or functional divergence of duplicated genes[72]. Vidal et al.[73] found that, in some cases, apparently a homolog of C. canephora is recruited to be expressed in certain tissues, while *C. eugenioides* homologs are silenced. On this way, differences in expressions in *C. arabica* can be attributed to different sub-genomes of the ancestors of *C. canephora* or *C. eugenioides*. These genes may be good candidates for future characterizations that would help to understand regulation processes during development and maturation of fruits in Coffea ssp.

The majority of the ERFs have demonstrated an increase in plant tolerance to biotic and abiotic stresses [33, 75, 76]. In this work, the ERFs *CaERF*06, *CaERF*07, *CaERF*11 presented high expression in libraries subjected to water stress. These genes belong to the DREB subfamily, which play an important role in plant tolerance to abiotic stress, recognizing the Dehydration Responsive Element (DRE), the *core motif* A/GCCGAC [33]. Studies have showed that the overexpression of DREB genes in Arabidopsis activate the expression of several genes related to stress, thus improving the tolerance to drought, salinity and low temperature [33, 77, 78]. For example, the overexpression of the *AoDREB* gene of *Asparagus officinalis* L in transgenic Arabidopsis induced the expression of genes rd29A and COR15A, resulting in higher tolerance of transgenic plants to drought and salinity [79]. On this way, these genes are promising for further studies that will help to understand the regulation mechanisms of the ERF family related to responses of *C. arabica* to different stresses.

Previous work suggests the hypothesis that a group-specific expression profile is occurring. For example, from 8 genes belonging to the Group VII, 5 are expressed in fruits, where the ERFs *CaERF*14, *CaERF*15 and *CaERF*18 present a high relative expression. This group has been associated with fruit maturation. *LeERF*2 in tomato [53], *MdERF*1 in apple [87], *PsERF2a* and *PsERF2b* in plum [31] and *AdERF10* and *AdERF14* in kiwi [69] are proteins that are expressed during the maturation of fruits belonging to Group VII. The ripening induction was also related in the Group VIII in plum [80] and grape [15,39]. Other works have demonstrated that members of the Group IX present induction of expression when subjected to diverse pathogen attacks. Constitutive overexpression of the *AtERF*2 of the IXa subgroup probably induced the gene expression of the PDF1.2 gene [81]. On the same way, the overexpression of the *AtERF1* gene in Arabidopsis, a homolog next to *AtERF2*, gives resistance to *Botrytis cinerea, Sclerotinia sclerotiorum* and *Erysiphe orontii* in Arabidopsis [26]. Anderson et al. [82] demonstrated that the overexpression of the *MtERF*1-1 gene in roots of *Medicago truncatula* increased the resistance to *Rhizoctonia solani*, as well as to *Phytophthora medicaginis*. Thus, the genetic profile expression suggests a functional level of specialization for the investigated ERF Groups, although it is expected a high degree of overlapping functions in large plant genes families [83, 84, 85]. On this way, the presence of distinct expression profiles of the ERFs observed in *C. arabica* by *in silico* analysis may be associated to the phylogenetic distance among sequences, that is, the ERF phylogenetically related proteins have more similar patterns of expression than the divergent sequences.

The ERF gene family plays a crucial role in the development regulation, as well as in the responses to abiotic and biotic stresses. With the sequenced transcriptome of *C. arabica* by a Brazilian consortium (Brazilian Coffee Genome Project), 36 ERFs were identified in *C. arabica* in this work, where 12 ERFs belong to the DREB subfamily and 24 to the ERF subfamily. The gene expression profiling showed high levels of expression in libraries derived from tissues of fruits, leaves, and flowers and libraries subjected to water stress. From the comparison of the homologs with other species, whose genome was sequenced together with expression profiles, it is suggested that the ERFs of *C. arabica* are involved in different biological functions mediated by ethylene as control of development, maturation, and responses to water stress. *C. arabica* is a perennial species whose fruits have commercial value. Knowledge on the role of the ERF transcription factors in processes of development and maturation of this species opens opportunities for investments in plant breeding programs to increase the production and the coffee bean quality.

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ARTICLE

Typical Correlation Analysis between Forage Type Triticale Production Performance and Different Pilot Ecological Factors

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ABSTRACT

Domestic and foreign researches on triticale mainly focus on hereditary traits and excellent characteristics, but there are few reports on triticale yield and quality in the severe cold pastoral areas of Gansu and Qinghai. In this study, Gannong 2 triticale cultivars have been bred and planted in typical ecological areas according to the characteristics of different ecological regions. By studying the relationship between quality traits and various ecological factors, the effects of different ecological factors on the quality of Triticale hay have been clarified, which provides a reasonable basis for future triticale breeding and large-scale and targeted planting layout. The production performance and nutritional qualities of Triticale Trial in Maqu County, Gansu Province and Gannong No. 2 in Dulan County, Qinghai Province are obviously superior to other pilot sites. Through reasonable fertilization, the production performance and nutritional quality of triticale are the best. Triticale production performance is significantly related to climatic factors in different pilots. The most suitable planting area for Gannong 2 is Maqu County, Gansu Province.

1. Introduction

Animal husbandry is a pillar industry in the alpine pastoral area of the Qinghai-Tibet Plateau, and the development status of animal husbandry directly affects the living standards of the people in the pastoral area [1]. In the past 20 years, with the impact of global warming and human activities, grassland in alpine pastures has been degraded on a large scale, and the output of forage grass has been continuously reduced, resulting in a gradual decline in the economic development of animal husbandry in alpine pastures [2]. Triticale has the characteristics of barren tolerance, drought tolerance, cold resistance, and disease resistance [3]. In alpine pasturing areas with variable climates and poor water and fertilizer conditions, it can show its stable yield advantage. The yield is higher than that of rye. Elymus spike and oats [4], so large-scale planting of forage triticale in the alpine pasturing area can just solve the problem of insufficient forage production in the alpine pasturing area. At present, researches on the nutritional

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quality of triticale at home and abroad mostly focus on genetic traits and excellent characteristics [4-6], and there is no research on the relationship between ecological factors and the quality of triticale hay. This study first studied the production performance and nutritional quality of triticale in Different test points. Next use SPSS software to perform a correlation analysis of the ecological factors and the production performance and nutritional quality of triticale at 4 test sites, and finally clarify the effects of different ecological environmental factors on the yield and quality of triticale hay. Influence, provide a reasonable basis for future triticale breeding and large area planting.

2. Materials and Methods

2.1 Overview of the Test Site

Maqu County is affiliated to Gannan Tibetan Autonomous Prefecture in Gansu Province, located in the southwest of Gannan Tibetan Autonomous Prefecture in Gansu Province, at the eastern end of the Qinghai-Tibet Plateau, at the junction of Gansu, Qinghai, and Sichuan Provinces. Hezuo City is located in the northern part of Gannan Tibetan Autonomous Prefecture. It is of high-cold and humid type, with long cold seasons and short warm seasons. The average annual temperature is minus 0.5 ℃ -3.5 ℃ , the extreme maximum temperature is 28 ℃ , and the extreme minimum temperature is -23 ℃ . The average annual precipitation is 545 mm, concentrated in July, August and September. The cooperation area has an average frost-free period of 48 days, and the main natural disasters are frost, hail and rain. The sunshine is abundant throughout the year and the solar energy utilization rate is high. The surface runoff is 200-350 mm deep and the annual evaporation is 1222 mm.

Dulan County is located in the southeast of Haixi Mongolian-Tibetan Autonomous Prefecture in Qinghai Province. The plateau has a dry continental climate with long cold seasons and short warm seasons. The annual average temperature is 1.4-5.1 ℃ , the lowest extreme temperature is -29.8 ℃ , and the highest extreme temperature reaches 33 ℃ . The average annual precipitation is 179.1 mm, concentrated in June, July and August. The average altitude is 3180 m; the annual evaporation is 1358-1765 mm; and the annual sunshine hours are 2903.9-3252.5 hours.

Guide County is located in the southeast of Hainan Tibetan Autonomous Prefecture in Qinghai Province. It has a plateau continental climate with long sunshine hours and strong solar radiation. Spring is dry and windy, summer is short and cool, autumn is wet and rainy, winter is long and dry, and the temperature varies greatly from day to day. The annual average temperature is 7.2 ℃ , the extreme highest temperature over the years is 34 ℃ , and the extremely low temperature is -23.8 ℃ . The annual average precipitation is 251-559 mm, the annual frost-free period is 258 days, the crop growth period is 223 days, and the annual sunshine hours are 2928 hours.

2.2 Test Materials

Main material: Gannong No. 2 triticale.

2.3 Experimental Design

The test was carried out at 4 test points in Guide (E1), Dulan (E2), Hezuo (E3) and Maqu (E4). The 4 experimental sites have the same experimental design, random block design, drilled sowing, row spacing 20 cm, sowing depth 3-5 cm, sowing amount calculated according to 7.5 million seedlings·hm-2, 3 repeats, plot area 15 m^2 (5 m×3 m). Plant protection rows 1 m around the test site. No fertilizer was applied to the 4 test sites, and weeds were timely controlled during the test period.

2.4 Measurement Indicators and Methods

(1) Grass production: during the flowering period [3]. All the above-ground parts of the plants in each plot are cut and weighed to obtain the yield of fresh grass. At the same time, 500 g were sampled and air-dried naturally to a constant weight. The yield of hay was weighed to calculate the fresh-dry ratio. Calculate the hay yield of each plot based on the fresh-dry ratio.

(2)Number of branches: before cutting during flowering period. Randomly select 1 m samples in each plot (except for the side rows), and count the number of branches with plant height higher than 50 cm in the sampling section [3].

(3)Plant height: before cutting during flowering period. Randomly select 10 individual plants in each plot, and measure the natural height from the ground to the highest point. The average of 10 plants is used as the plant height of triticale in this area [3].

(4)Nutritional value: Use a pulverizer to crush the dried grass samples, pass through a 1 mm sieve, randomly select 3 samples from the uniformly mixed grass samples, and measure various indicators in parallel. The content of crude protein (CP) is determined by Kjeldahl method [7], the neutral detergent fiber (NDF) and acid detergent fiber (ADF) are analyzed by Fan's detergent fiber analysis method [7], and the in vitro digestibility (DMD) is artificial Rumen method [7], crude fat (EE) using Soxhlet fat extraction method [7].

(5)Soil nutrients: The collected samples are placed in the room to air dry, the air-dried soil samples are ground, and the samples are pre-treated. The soil available nitro-

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gen (AN) content is extracted by potassium chloride-flow injection analyzer Available phosphorus content (AP) adopts sodium bicarbonate extraction-molybdenum antimony colorimetric method, soil available potassium content (AK) adopts ammonium acetate extraction-flame photometer method, soil organic matter (OM) adopts potassium dichromate volumetric method , The pH value of the soil adopts the 2.5:1 water-soil ratio acidity meter method [8].

(6)Temperature and rainfall: data comes from 2018 data from the National Meteorological Science Data Sharing Service Platform.

2.5 Data Processing

Use Microsoft Excel 2010 software to organize the test data, and then use SPSS 26.0 software to analyze and process the test data.

3. Results and Analysis

3.1 Comparison of the Production Performance Differences of Triticale in Different Pilots

Table 1. Comparison and Analysis of Production Performance of Triticale in Different Pilot Plants

Factor	Hay yield (Kg/hm^2)	Number of branches (Ten thousand branches/hm^2)	Plant height (cm)
Experiment site	10.77**	163.72**	45.75**

*Note: ** indicates significant difference at the 0.01 level, and * indicates significant difference at the 0.05 level.*

It can be seen from Table 1 that there are extremely significant differences in plant height, hay yield, and number of branches in different trials, and multiple comparisons are required. See Figure 1 to Figure 2 for details.

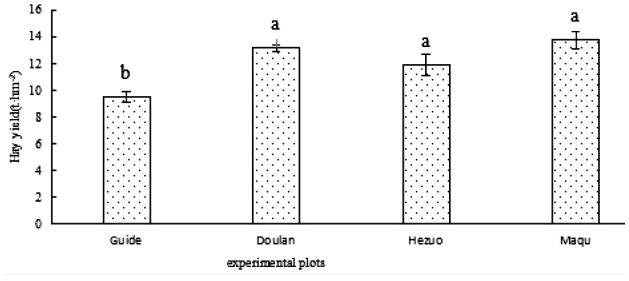

Figure 1. Multiplicity comparison of hay yield differences triticale in different experimental plots

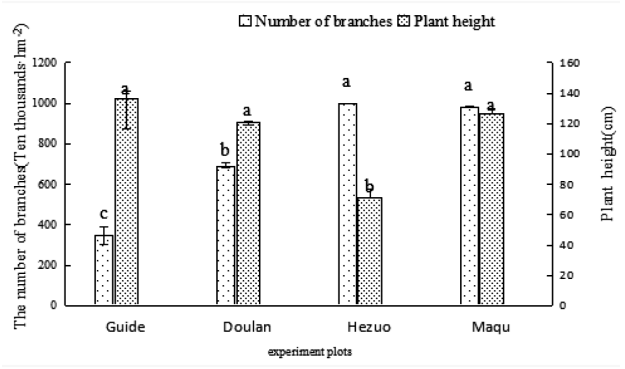

Figure 2. Comparison of the number of branches and plant height in different experimental sites

3.2 Comparison of Differences in Nutritional Quality of Triticale in Different Pilots

Table 2. Variance Analysis of Nutritional Quality of Triticale Gannong No.2 in Different Trials

Factor	CP(%)	NDF(%)	ADF(%)
Experiment site	25.34**	6.31**	4.38*

It can be seen from Table 3 that there are very significant differences in the neutral detergent fiber, acid detergent fiber and crude protein of triticale in different trials, and multiple comparisons are needed. See Figure 3 for details.

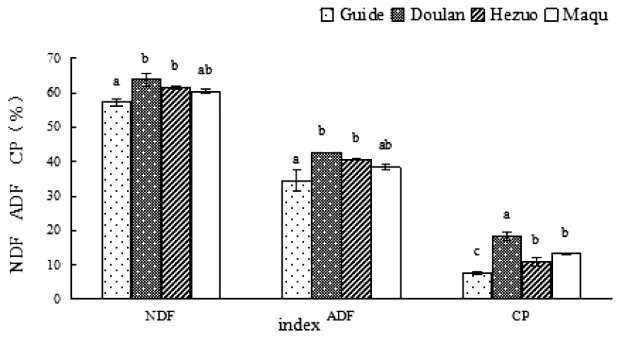

Figure 3. Difference analysis of nutrition indicators in different experimental sites

From Figure 3, it can be seen that Guide's triticale has the lowest neutral detergent fiber and acidic detergent fiber, which is not significantly different from Maqu's point, and is significantly lower than Dulan's and Hezuo's points; Dulan's triticale has the highest crude protein content. Significantly higher than the other 3 pilots, the Maqu spot's triticale protein content is second, and there is no

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significant difference from the cooperation spot, which is significantly higher than the Guide spot.

3.3 Correlation between Soil Nutrients and Production Performance, Nutritional Quality of Triticalein Different Pilot Sites

Table 3. Correlation between Soil Nutrients and production performance ,Nutritional Quality of Triticalein Different Pilot Sites

Factor	Plant Height (cm)	Hay yield (kg/hm^2)	Number of branches (Ten thousand branches//hm^2)	NDF(%)	ADF(%)	CP(%)
OM	0.44	0.81*	0.36	0.38	0.29	0.65
AN	-0.35	0.95**	0.76	0.92**	0.90**	0.87*
AP	0.39	0.85*	0.34	0.56	0.47	0.81*
AK	0.28	-0.83*	-0.53	-0.99**	-.97**	-0.96**
PH	0.09	-0.47	-0.68	0.15	0.143	0.15

*Note: ** indicates significant difference at the 0.01 level, and * indicates significant difference at the 0.05 level.*

The canonical correlation analysis of soil nutrients, triticale production performance and nutritional quality indicators in different pilots was carried out. The results are shown in Table 3. Triticale plant height, number of branches and soil nutrients are significantly uncorrelated; Triticale hay yield is positively correlated with soil organic matter, available nitrogen, and available phosphorus, and is significantly negatively correlated with available phosphorus; neutral detergent fiber of triticale. Acid detergent fiber has a very significant positive correlation with available phosphorus, and a very significant negative correlation with available potassium; the crude protein content of triticale is significantly positively correlated with available nitrogen and available phosphorus, and has a very significant negative correlation with available potassium.

3.4 Correlation between Climate Factors and Nutritional Quality of Triticale in Different Pilot Sites

Correlation analysis of different pilot climatic factors and Gannong No. 2 triticale nutrition indicators is carried out, and the results are shown in Table 4. It can be seen from Table 4 that the hay yield of Gannong No. 2 triticale has a significant negative correlation with the effective accumulated temperature; the number of branches of Gannong No. 2 triticale is significantly negatively correlated with the effective accumulated temperature and rainfall; the remaining Gannong No. 2 Xiaohei Wheat plant height, hay yield, acid detergent fiber, neutral detergent fiber, crude protein and other indicators are positively or negatively correlated with effective accumulated temperature and rainfall, but they are not significant.

Table 4. Correlation between Climate Factors and Nutritional Quality of Triticale in Different Pilot Sites

Factor	Plant height (cm)	Hay yield (kg/hm^2)	Number of branches (Ten thousand branches/hm^2)	NDF (%)	ADF (%)	CP (%)
Effective accumulated temperature	-0.07	-0.83*	-0.72*	-0.27	-0.24	-0.38
Rainfall	0.53	-0.50	-0.89**	-0.09	-0.15	0.09

4. Discussion

4.1 Comparison of Differences in Production Performance and Nutritional Quality of Triticale in Different Pilots

With the continuous development of herbivorous animal husbandry in my country, forage grass has gradually shown a trend of in short supply and uneven distribution [8-9]. My country's natural grassland has been declining due to long-term overgrazing and overexploitation, and the productivity of forage grass has declined sharply, which has not met the sustainable development requirements of herbivorous animal husbandry [10-11]. When a new germplasm appears, it is particularly important to play its production potential and screen out the most suitable areas for planting [3]. Based on the comparison of the production performance and nutritional value of Gannong No. 2 in different pilots, this article shows that the production performance and nutritional quality of triticale in Maqu area of Gansu Province and Dulan area of Qinghai Province are the best, and they are most suitable for Gannong No. 2 growth Area.

4.2 Correlation Analysis of Soil Nutrients in Different Pilots with Triticale Production Performance and Nutritional Quality

In order to solve the large-scale increase in forage productivity, to meet the problems of insufficient and un-

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balanced forage supply in different regions. Through research, it is found that the yield of triticale hay is positively correlated with soil organic matter, available nitrogen, and available phosphorus, and has a significant negative correlation with available phosphorus; neutral detergent fiber, acid detergent fiber and available phosphorus are extremely significantly positively correlated. It is extremely significantly negatively correlated with available potassium; the crude protein content of triticale is significantly positively correlated with available nitrogen and phosphorus, and is extremely significantly negatively correlated with available potassium. Therefore, the production performance and nutritional quality of triticale can not be improved only by the amount of fertilizer, but the production performance and nutritional quality of triticale can be optimized through a reasonable amount of fertilizer. This is also basically consistent with the conclusion of the optimization screening of triticale cultivation conditions [12].

4.3 Correlation Analysis of Different Pilot Climatic Factors and Nutritional Quality of Triticale

The canonical correlation analysis was carried out on the climatic factors of different pilots and the nutritional indicators of Gannong No. 2 triticale. It was found that Gannong No. 2 triticale hay yield was significantly negatively correlated with the effective accumulated temperature, and the number of branches of Gannong No. 2 triticale was significantly negatively correlated with effective accumulated temperature and rainfall. This is because the production performance of triticale is significantly related to different pilot climate factors, which is basically consistent with the conclusion that the stability of production performance and ecological adaptability of different triticale genotypes are very different [13].

5. Conclusions

In this experiment, the plant height, hay yield, number of branches, neutral detergent fiber, acid detergent fiber, crude protein and other production performance and nutritional indicators of Gannong No. 2 triticale planted in different pilots were compared. The results showed that the production performance and nutritional quality of triticale in Maqu County of Gansu Province and Gannong No. 2 Triticale in Dulan County of Qinghai Province were significantly better than other trials.

Through the study of the correlation analysis between soil nutrients and the production performance and nutritional quality of triticale in different pilots, the production performance and nutritional quality of triticale can not be improved only by the amount of fertilizer, but by the reasonable amount of fertilizer, the production of triticale The best performance and nutritional quality.

By studying the typical correlation analysis of different pilot climatic factors and the nutritional quality of triticale, it can be seen that the production performance of triticale is significantly related to the climatic factors of different pilots.

Foundation Item

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