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Genetic diversity, agronomic potential and reaction to downy mildew in genotypes of biofortified mini lettuce

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ABSTRACT. Lettuce is one of the most consumed leaf vegetables in the world, highlighting itself as a novelty in the market as mini type cultivars. During the winter the downy mildew can lead to severe damage. Thus, the objective of this work was to evaluate the genetic diversity, agronomic potential and downy mildew reaction in biofortified mini lettuce genotypes. The experiment was carried out in the University of Uberlandia, Monte Carmelo Campus. The statistical method used for the field experiment was the randomized complete block design, with 19 treatments (17 genotypes of the generation F3:4 and two cultivars of commercial lettuce) and two repetitions. The following parameters were evaluated: crown diameter (cm); rod diameter (mm); number of leaves per plant and spad index. The data were submitted to multivariate analyses were performed. The genetic divergence was represented by dendrogram obtained by the hierarchical UPGMA method and by the Tocher optimization method. The laboratory experiment was carried out in a completely randomized design with 18 genotypes, two Bremia lactucae breeds (3 and 4) and three repetitions. In order to select resistant genotypes, inoculations were carried out, using distilled water mixture with the sporangia of the pathogen and Tween 20. The monitoring was performed daily and when the first sporulation appeared on cotyledonary leaves of the susceptible cultivar, the genotypes were evaluated, verifying the presence or not of sporulation and necrosis. The use of Mahalanobis distance $D^2_{ii'}$ as a measure of dissimilarity allowed the formation of two groups by the Tocher method. The grouping of the accesses by the UPGMA hierarchical method formed four groups, with the cut-off point established at the 7% similarity distance. The all lettuce genotypes F3:4 showed low percentage of necrotic plants and sporulated plants.

Keywords: *Lactuca sativa*; Bremia lactucae; Resistance; Vitamin A; Tocher method; Hierarchical method UPGMA

INTRODUCTION

The lettuce (*Lactuca sativa* L.) is the most consumed leafy vegetable in the world and the third crop for production volume (ABCSEM, 2013). The cultivation of this specie annually moves, on average, two million dollars only in retail, with a production of more than 1.5 million tons per year (ABCSEM, 2015). Mostly, the cultivation takes place by family producers and can generate five direct jobs per hectare (Sala et al., 2008).

The loose leaf lettuce group leads the market in Brazil (Sala and Costa, 2012), However, the characteristics of Brazilian families have changed in relation to the composition of the number of individuals and the requirement in quality, which has led to the need to produce vegetables that meet the demand of the consumer market in differentiated products. With this in mind, companies have introduced constant innovations, such as mini vegetables and baby leaves or baby leaf, which can stimulate consumption by the population, especially by children, who are attracted to small products (Purquerio and Melo, 2011).

The mini lettuce is considered a differentiated product due to the reduced size of the leaves, color varieties, excellent flavor and texture, and long shelf life (Takahashi and Cardoso, 2014). Allied to this, leafy vegetables are important sources of vitamins and minerals, which may be involved in pro-vitamin and antioxidative activities (Oliveira et al., 2005). Although the lettuce has high nutritional value, the present cultivars present low content of carotenoids, a type of precursor of vitamin A. Recently, numerous beneficial effects of carotenoids have been recognized, notably the prevention of some types of cancer, heart disease and muscular degeneration, which has stimulated intense research on the role of these compounds such as antioxidants and as immune response regulators (Uenojo et al., 2007).

Because lettuce is one of the most present foods in the Brazilian diet, any strategy aimed at enhancing its nutritional quality can result in several health benefits. One strategy would be biofortification, aiming to increase the content of pro-vitamin A. There are reports that the genetic control of carotenoids in lettuce has high heritability (84%) and can therefore be enhanced through classical plant breeding (Cassetari et al, 2015). However, the production of this vegetable is limited by the attack of numerous pests and diseases, especially the pathogen Bremia lactucae Regel (*B. lactucae*), the etiologic agent of lettuce downy mildew. The downy mildew stands out as one of the most important and severe diseases of the crop during the winter due to its high destructive power (Nunes et al., 2016). The disease is difficult to control dues to the existence of great variability of *B. lactucae* breeds, that is, innumerable variations of virulence, which can be distinguished only by the joint reaction of differentiating cultivars (Van Ettekoven and Van Der Arend, 1999). However, research shows that it is possible to obtain lettuce genotypes with resistance to downy mildew (Castoldi, et al., 2014). Thus, the objective of this work was to evaluate the genetic diversity, the agronomic potential and the downy mildew reaction in biofortified mini lettuce genotypes.

MATERIALS AND METHODS

The experiment was carried out in the field and in the laboratory. The field experiment was conducted from August 2016 to January 2017, at the Horticultural Experiment Station of the Federal University of Uberlândia, in the municipality of Monte Carmelo-MG (873 m altitude, 18º42’43,19”S e 47º29'55,8” W), whose climate is temperate humid, with hot summers and dry winters, according to the climatic classification of Köppen. The statistical design was a randomized complete block design, with 19 treatments and two replications. Each plot was composed of 16 plants (1.44 m^2/plot), being considered for evaluation the four central plants. The spacing used was 30 × 30 cm.

The treatments consisted of the genotypes from the fourth self-fertilization of the hybridization between Uberlândia 10.000 versus cultivar Belíssima (UFU-66#2, UFU-66#3, UFU-66#4, UFU-66#7, UFU-66#8, UFU-104#1, UFU-104#6, UFU-215#1, UFU-215#2, UFU-215#3, UFU-215#4, UFU-215#6, UFU-215#7, UFU-15#10, UFU-215#12, UFU-215#13, UFU-215#14), besides two commercial cultivars (Belíssima and Uberlândia 10.000). The cultivar Uberlândia 10,000 is a cultivar of lettuce with high content of carotenoids (Sousa et al., 2007) and smooth and susceptible to downy mildew. Already, the cultivar Belíssima is characterized by plants of medium size and leaves of high crespicide, intense red color and resistance to downy mildew (Tecnoseed, 2017).

The seeding was carried out in 200 cell polystyrene trays filled with commercial coconut fiber substrate. These were kept in a greenhouse (7 m × 4 m) covered with 150 micra transparent anti UV plastic. 35 days after sowing, the seedlings were transplanted in beds previously prepared and fertilized, according to soil analysis and crop recommendations. The plant fertilization consisted of 150 kg ha^{-1} of N, 60 kg ha^{-1} of K_2O and 100 kg ha^{-1} of P_2O_5. This total was applied to planting and covering. The covering fertilization were applied at 15, 30 and 40 days after transplantation, totaling 120 kg ha^{-1} of N and 48 kg $ha^{-1}$of K_2O (Ribeiro et al., 1999). The irrigation sprinkler system was used with a single flow rate of 0.45 m^3 h^{-1}, spaced with 12.0 × 12.0 m. When the plants reached their maximum vegetative development, that is, 35 days after the transplant to the field, the following evaluations were carried out: crown diameter (cm); rod diameter (mm); number of leaves per plant and spad index (using the chlorophyllometer model Monolta SPAD-502 CFL1030).

The data were submitted to analysis of variance by the F test (p=0.05). The averages were compared by the Scott-Knott test (p=0.05). Subsequently, multivariate analyzes were performed to determine the genetic dissimilarity between the genotypes, obtaining the matrix of dissimilarity by the generalized Mahalanobis distance ($D^2_{ii'}$). The genetic divergence was represented by dendrogram, obtained by the hierarchical method Unweighted Pair-Group Method Using Arithmetic Averages (UPGMA) and by the Tocher optimization method. The validation of the grouping by the UPGMA method was determined by the co-phenotype correlation coefficient (CCC), calculated by the Mantel test (1967). The relative contribution of the quantitative traits was calculated according to Singh (1981).

To establish the cut-off point in the dendrogram, points of abrupt level change were used as reference (Sudré et al., 2005). All the data obtained were analyzed using the software Genes v. 2015.5.0 (Cruz, 2013). The laboratory experiment was conducted at the Laboratory of Analysis of Seeds and Genetic Resources (LAGEN), located at the Federal University of Uberlandia, Monte Carmelo Campus. The statistical design used was the randomized complete block design, with 17 genotypes, two *B. lactucae* breeds (3 and 4) and three repetitions. Each plot was composed of 30 plants, being evaluated all the plants of the plot. The breeds 3 and 4 of *B. lactucae* were selected because they were races of great occurrence in Brazil (Souza et al., 2011, Castoldi et al., 2012, Galatti et al., 2012; Nunes et al., 2016). To select 100% resistant genotypes, 30 seeds of each generation genotype F3:4 (UFU-66#2, UFU-66#3, UFU-66#4, UFU-66#7, UFU-66#8, UFU-104#1, UFU-104#6, UFU-215#1, UFU-215#2, UFU-215#3, UFU-215#4, UFU-215#6, UFU-215#7, UFU-15#10, UFU-215#12, UFU-215#13, UFU-215#14) and of the cultivar Solaris (control) were seeded separately in transparent plastic box of the gerbox type (11 × 11 × 3.5 cm), lined with germitest paper moistened and maintained for 15 days in incubation chamber type BOD at the temperature of 13°C and 12 h photoperiod.

After this period of 15 days, the inoculation was performed in each genotype, separately, using a suspension containing *B. lactucae* sporangia, races 3 and 4. The inoculation was performed according to the technique of Ilott et al. (1987), modified by Franco (2016). For this purpose, lettuce leaves containing sporangia from each *B. lactucae* breed were cut; with the aid of distilled water and surfactant (Tween 20), to increase sporangium adhesion in the cotyledonary leaves of the inoculated genotypes. The suspension was shaken on a Vortex shaker and the suspension was adjusted under stereomicroscope to 5×10^4 mL^{-1} sporangia. In the inoculation of the suspension Pauster-type pipettes were used for each isolate, so that the solution was deposited in the cotyledonary leaves of each genotype until the point of drainage. Afterwards, the boxes were reallocated in BOD with a temperature of 13°C, and during the first six hours they were left in a darkroom and after that time the photoperiod was adjusted to 12 hours.

The plants monitoring was daily, and when the first sporulation appeared on the susceptible cultivar Solaris, which normally ranged from 12 to 15 days, the F3:4 genotypes were evaluated individually and the presence or absence of sporulation and necrosis was verified, according to methodology proposed by Van Ettekoven and Van Der Arend (1999). This methodology is based on the placement of +, (+), - or (-) signals according to the percentage of tissue damage levels of lettuce plants, being placed: +: when more than 80% of the seedlings present sporulating lesions; (+): when more than 80% of the seedlings present necrotic spots and with many sporulating lesions; -: when less than 5% of the seedlings present sporulating lesions; e (-): when the seedlings present necrotic spots and with few sporulating lesions. Based on the mean of each evaluated trait, the genotypes that showed signs - or (-) were considered resistant.

RESULTS

There was a significant difference (F test, 5% probability) for all characters analyzed (spad index, plant diameter, stem diameter and number of leaves per plant). The genotypes UFU-215#14, UFU-215#2, UFU-215#1 and UFU-215#12 stood out in terms of chlorophyll content, with an average of 184.06; 181.38; 181 and 172.63%, respectively, more than the cultivar Belíssima (Table 1).

Table 1. Mean of carotenoid content (SPAD index), plant diameter (cm), stem diameter (mm) and number of leaves per plant.

Genotypes	SPAD Index	Plant Diameter (cm)	Stem Diameter (mm)	Number of Leaver per Plant
UFU-66#2	26,19 e	22,35 c	19,99 c	35,20 b
UFU-66#3	25,33 e	19,75 d	22,52 b	45,60 a
UFU-66#4	30,09 d	22,30 c	21,01 c	34,90 b
UFU-66#7	24,23 e	21,40 c	25,84 a	44,20 a
UFU-66#8	24,84 e	21,45 c	23,47 b	40,40 a
UFU-104#1	32,03 d	21,75 c	21,60 c	32,90 b
UFU-104#6	26,55 e	21,55 c	23,78 b	41,50 a
UFU-215#1	44,96 a	25,70 b	23,01 b	37,30 b
UFU-215#2	45,02 a	24,55 b	25,97 a	37,70 b

UFU-215#3	30,51 d	22,40 c	26,49 a	37,50 b
UFU-215#4	41,40 b	29,05 a	24,56 a	36,80 b
UFU-215#6	37,86 c	14,55 e	18,97 c	23,20 d
UFU-215#7	39,75 b	21,05 c	22,44 b	32,00 c
UFU-215#10	40,97 b	18,60 d	18,94 c	28,80 c
UFU-215#12	43,62 a	24,90 b	20,95 c	38,60 b
UFU-215#13	36,04 c	18,45 d	17,71 c	30,30 c
UFU-215#14	45,45 a	23,50 c	19,65 c	29,60 c
Belíssima	16,13 f	14,80 e	11,87 d	13,90 e
Uberlândia 10,000	16,22 f	25,45 b	24,01 b	37,90 b
CV (%)	6,43	10,91	15,16	16,99

*Means followed by distinct letters in the column differ from each other by the Scott-Knott test at 5% probability.

As for plant diameter, the highest value was obtained for genotype UFU-215#4, being 96.28% higher than the diameter of the cultivar Belíssima, which is not considered mini lettuce (Table 1). For stem diameter, all genotypes evaluated differed significantly from commercial cultivars Belíssima, with emphasis on UFU-215#3, UFU-215#2, UFU-66#7 and UFU-215#4 genotypes. Which showed to be 123.17; 118.79; 117.69 and 106.91% respectively higher (Table 1).

For the number of leaves per plant, all genotypes showed values close to or higher than 30, with the exception of genotype UFU-215 # 6, which presented the least amount of leaves among the genotypes of mini lettuce evaluated (23, 20), however, its leaf number was 66.91% higher than the commercial cultivar Belíssima (Table 1).

The use of Mahalanobis distance $D^2_{ii'}$ as a measure of dissimilarity allowed the formation of two groups by the Tocher method (Table 2). The group I was formed by 89.5% of the analyzed genotypes (UFU-66#4; UFU-104#6; UFU-215#12; UFU-215#10; UFU-104#1; UFU-215#14; UFU-215#13; UFU-66#3; UFU-66#8; UFU-66#7; UFU-66#2; UFU-215#4; UFU-215#3; UFU-215#2; UFU-215#1; UFU-215#7 and Uberlândia 10,000)The group II was formed only by genotypes UFU-215#6 and Belíssima.

Table 2. Representation of the cluster generated by the Tocher Optimization Method based on Mahalanobis distance, estimated from four agronomic characteristics, analyzed in 19 mini lettuce genotypes.

Groups	Genotypes
I	1006 66#8 66#7 66#3 215#3 66#4 1001 66#2 215#7 UDIA 215#12 215#1 215#2 215#4 215#10 215#13
II	215#6 Belissima
Total	19

UDIA = Uberlândia 10,000

The grouping of the accesses by the UPGMA hierarchical method formed four groups (Figure 1), with the cut-off point established at the 7% similarity distance, established at the site of abrupt change in the ramifications present in the dendrogram (Sudré et al., 2005). The group was formed by the genotypes UFU-66#8, UFU-215#1, UFU-215#14, UFU-215#4, UFU-215#12 and Belíssima. The group II was formed by the genotypes UFU-66#2, UFU-215#6, UFU-104#6, UFU-66#3 e UFU-104#1. The group III was formed just by one genotype UFU-215#2 and the group IV was formed by the genotypes UFU-215#7, Uberlândia 10,000, UFU-66#4, UFU-215#10, UFU-215#13, UFU-66#7 and UFU-215#3. The co-expressed correlation coefficient

(CCC) was 89.86% and it was found to be significant in the t-test (p <0.01), showing consistency of the grouping.

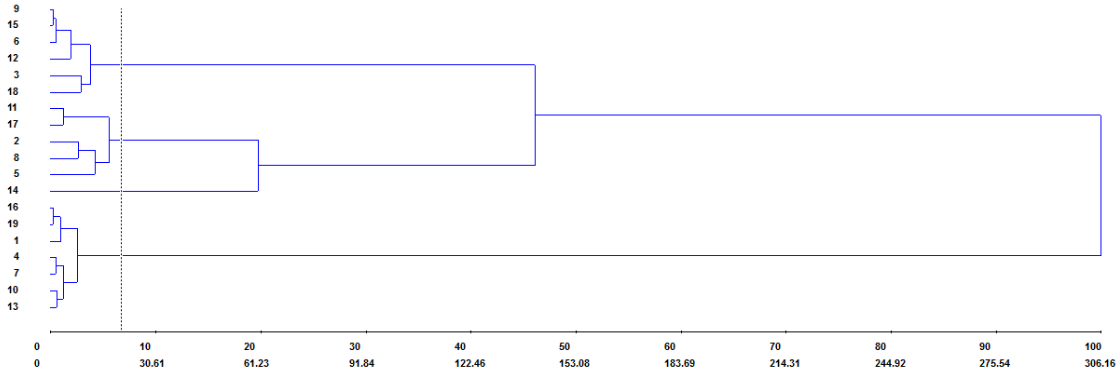

Figure 1. Dendrogram of genetic divergence among 19 genotypes of mini lettuce, obtained by the Unweighted Pair-Group Method Using Arithmetic Averages "UPGMA" as a measure of dissimilarity. The numerals indicate the genotypes UFU. 1=UFU-66#4; 2=UFU-104#6; 3=UFU-215#12; 4=UFU-215#10; 5=UFU-104#1; 6=UFU-215#14; 7=UFU-215#13; 8=UFU-66#3; 9=UFU- 66#8; 10=UFU-66#7; 11=UFU-66#2; 12=UFU-215#4; 13=UFU-215#3; 14=UFU-215#2; 15=UFU-215#1; 16=UFU-215#7; 17=UFU-215#6; 18=Belíssima and 19=Uberlândia 10,000.

The characteristic SPAD index was responsible for the greater relative contribution of divergence among the genotypes (38.03%). On the other hand, the variable number of leaves contributed only with 19.78%. It is observed that after the resistance test, all genotypes of biofortified miniature lettuce from the fourth self-fertilization of the cross between Uberlândia 10,000cv. Belíssima showed low percentage of necrotic plants (variation from 0 to 25.56% and from 0 to 60.0%, respectively for the races 3 and 4) and low percentage of sporulated plants (variation from 0 a 7.78% and from 0 to 4.40%, respectively for the races 3 and 4) (Table 3).

Table 3. Reaction of the genotypes of mini lettuce to *B. lactucae*, races 3 and 4.

Genotypes	*B. lactucae*, race 3			*B. lactucae*, race 4		
	PN (%)	PE (%)	R or S	PN (%)	PE (%)	R or S
UFU-66#2	12,23	0	R	32,22	0,00	R
UFU-66#3	15,56	3,33	R	17,78	3,33	R
UFU-66#4	25,56	0,00	R	22,22	6,67	R
UFU-66#7	11,11	0,00	R	25,55	4,4	R
UFU-66#8	14,45	1,11	R	60,00	0,00	R
UFU-104#1	43,33	1,11	R	25,56	4,44	R
UFU-104#6	21,11	0	R	25,56	1,11	R
UFU-215#1	20,00	1,11	R	12,22	1,11	R
UFU-215#2	13,33	0,00	R	30,00	2,22	R
UFU-215#3	22,22	0,00	R	22,22	0	R
UFU-215#4	16,67	1,11	R	25,56	1,11	R
UFU-215#6	0,00	0,00	R	5,55	0,00	R
UFU-215#7	3,33	0,00	R	17,78	0,00	R
UFU-215#10	17,78	1,11	R	33,33	0,00	R
UFU-215#12	8,89	5,55	R	6,67	4,40	R
UFU-215#13	7,78	7,78	R	16,67	2,22	R
UFU-215#14	16,67	0,00	R	17,7,8	0,00	R

PN = Necrotic plants; PE = Sporulated plants; Susceptible (S) = when more than 80% of the seedlings presented necrotic spots and with many sporulating lesions; Resistant (R) = when less than 5% of the seedlings presented sporulant lesions and necrotic spots.

DISCUSSION

The non-destructive instantaneous analysis provided by the portable chlorophyllometer are an alternative indicator of the chlorophyll content present in the plant leaf (Klooster et al., 2012). All the genotypes of mini lettuce evaluated can be considered biofortified, since they presented relative superiority in relation to the chlorophyll content when compared to the control (Uberlândia 10,000), considered rich in beta-carotene (Sousa et al., 2007). There is a commercial preference for intermediate sized plants and reduced plant diameter, since this feature facilitates handling during cultivation and can avoid waste (Sala and Costa, 2012). When evaluating mini lettuce cultivars Takahashi and Cardoso (2014) verified that the diameter of the cultivars presented values close to 20 cm, which corroborates with the data obtained in the current experiment. These results are also similar to those obtained by Castoldi et al. (2012), evaluating different mini lettuce cultivars at different row spacing.

Santi et al. (2013), evaluating different progenies and lettuce cultivars observed values close to 30 leaves per plant. But Souza et al. (2008) obtained the highest number of leaves with cv. Regina (42.9). Similar data were obtained in the work in question, where it was possible to obtain cultivars of mini lettuce with higher amounts of leaves per plant. However, it is believed that this was only possible because the row spacing used was higher than that recommended for mini lettuce, which according to Castoldi et al. (2012), is 0.15 × 0.15 m.

The grouping obtained by the Tocher Optimization Method was not similar to the UPGMA, resulting in the formation of only two distinct groups, with the group II meeting the two genotypes (UFU-215#6 and Belíssima), which presented smaller plant diameters. On the other hand, with the adopted cut-off value (7%), the UPGMA hierarchical method formed four distinct groups, showing consistency of clustering by a significant correlation coefficient using the t-test ($p<0.01$). These results confirmed the existence of genetic diversity among the analyzed genotypes. The results confirmed the chlorophyll content, determined by the SPAD index, as the characteristic with greater relative contribution of the divergence between the genotypes. Nick et al. (2010) and Negreiros (2013) report the importance of defining the characters that have contributed significantly to genetic divergence, and those that may possibly be discarded in future works because they do not contribute significantly. The low percentage of necrotic plants and sporulated plants can be due to the fact that the cultivar Belíssima presents tolerance to *B. lactucae* (Tecnoseed, 2017), that is, probably present DM gene(s) or resistance factor(s) (FR). The DM (Downy mildew) genes or resistance factors (FR) have been widely used in lettuce cultivars, providing a high level of resistance to mildew (Castoldi et al., 2014). However they are effective temporarily, until new virulence genes occur within the pathogen population (Lebeda et al., 2007).

The low percentage of necrotic plants and sporulated plants in the F3:4 generation indicates the possibility of genetic control being due to a single gene with a complete dominance effect, as suggested by Araújo et al. (2014). Thus, the transfer of the gene or resistance factor, probably existing in the cultivar Belíssima, was transferred to the genotypes in question, during the self-fertilization procedures, which caused all the plants to show resistance. In addition, this high percentage of resistant plants may be due to the existence of a gene-to-gene interaction relationship in the Bremia genus, which results in an incompatible interaction between the pathogen and the plant, associated with a host hypersensitivity response, which blocks the infection of the disease and later the dispersion of the pathogen (Araújo et al., 2014). Braz et al. (2007) found that in the genetic control of lettuce for resistance to *B. lactucae* found in the Brazilian lettuce production fields different DM genes are involved, with the dominant allele being responsible for the resistance, for all the behaviors studied.

Castoldi et al. (2014) verified that among 69 progenies of the F2 generation with good agronomic characteristics, only 19 showed resistance (27.53% of the total plants), demonstrating that in a lettuce breeding program, the yield of plants with resistance to *B. lactucae* is low. In addition to this, resistance can be easily broken by the emergence of new strains of pathogenicity with different virulence characteristics (Souza et al., 2011; Castoldi et al., 2012). This factor, along with the difficulty of breeding lettuce, explains the scarcity of cultivars with resistance to *B. lactucae* in the Brazilian market.

CONCLUSION

In conclusion, there is genetic divergence between the genotypes of mini lettuce analyzed; all genotypes of mini lettuce are biofortified and with good agronomic characteristics; all evaluated lettuce genotypes present resistance to downy mildew, races 3 and 4.

CONFLICTS OF INTEREST

The authors declare no conflict of interest.

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