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Morphological, physiological, and biochemical indicators of quality in tobacco fruits and seeds

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ABSTRACT. Morphological or isozyme markers related to physiological maturation and deteriorative processes are important in the evaluation of seed quality. Two experiments were conducted to examine the possibility of using isozymes as indicators of quality in tobacco seed lots and fruit appearance as an indicator of physiological maturity in tobacco cultivars, based on the physiological and biochemical changes of the seeds. Cultivars CSC 444 and CSC 221 of tobacco fruits were harvested at various maturity stages and their physiological quality was assessed by germination, first count, germination speed index, time to reach 50% germination, cumulative average germination, and seedling emergence. We also assessed the activity of catalase (EC 1.11.6.1 – CAT), esterase (EC 3.1.1.1 – EST), isocitrate dehydrogenase (EC 1.1.1.41 – IDH), malate dehydrogenase (EC 1.1.1.37 – MDH), alcohol dehydrogenase (EC 1.1.1.1 – ADH), endo-β-mannanase (EC 3.2.1.78), and heat-resistant proteins during the process of maturation. Six lots of cultivar CSC 444 were used to differentiate the quality levels between the lots, and their characterization was determined by germination and vigor tests. In addition, we evaluated the enzymatic activity of CAT, EST, ADH, MDH, and heat-resistant proteins. During maturation of the fruits from the partially dark stage, we observed a progressive increase in germination and seed vigor. We concluded that appearance of the

fruit is an indicator of fruit maturity and quality in tobacco seeds. The enzymatic profile of ADH matches the physiological potential of the seeds, based on germination and emergence tests. Thus the ADH enzyme indicates the optimum stage to harvest fruits. In the EST and CAT enzymatic pattern analysis, we observed higher activity of these enzymes in lots with lower physiological quality. So the CAT and EST enzymes are biochemical indicators that can assess the deterioration of tobacco seed lots.

Key words: Isozyme markers; *Nicotiana tabacum;* Physiological maturity; Vigor

INTRODUCTION

Germination and vigor are important physiological attributes to be considered when assessing the quality of a lot in any seed production program. In general, factors such as the genetic material, the production environment, and fruit ripeness can positively or negatively affect seed quality (Moshatati and Gharineh, 2012). According to Eskandari (2012), the physiological stage when seeds are harvested affects the rate of decay and thus their conservation.

Quality is gradually achieved during seed development and is usually higher when there is maximum accumulation of dry mass (Olasoji et al. 2012); at this stage, seeds of most crops attain high germination and vigor rates, indicating physiological maturity (Queiroz et al.2011). An important aspect of seed technology is a need for indicators of physiological maturity or deterioration, which can be found in plants, fruits, and/or seeds and are related to seed quality.

For vigor evaluation, fast and reliable tests are needed to allow flexibility in the decision-making process, especially regarding harvesting, processing, storage, and marketing operations, thereby reducing risks and costs (Barbieri et al. 2012). However, when assessing the quality of tobacco seeds, it is difficult to carry out the traditional analysis tests because of their small size.

Electrophoresis has been used in isoenzyme studies to analyze not only changes in physiological seed quality, but also in genetic and biochemical regulations (International Seed Testing Association [ISTA], 1992). Since then, enzyme markers have been highlighted as valuable tools (Vidigal et al., 2009 and Tunes et al.,2011), because they assist in the identification process of the physiological state of seeds and may also help to understand factors that result in reductions in vigor and viability (Veiga et al., 2010).

Knowing the ideal harvest time defined by markers (morphological and/or biochemical) allows one to better plan the drying and processing operations (Sowmya et al., 2012), increasing the chances of obtaining high quality lots. After physiological maturity is attained, seeds are subject to degenerative changes of a physical, physiological, and biochemical nature that can result in reduced quality (Sediyama et al., 2012). Thus, one must understand the physiological maturation process to allow programming of the harvest (Alves et al., 2005).

In tobacco, *Nicotiana tabacum* (Solanaceae) unevenness in the flowering among plants or in the same plant affects the ripening of fruits, which is often reflected in the quality of the seed batches. Because of this peculiarity of tobacco seed production, it would

be ideal is to have a marker associated with fruit appearance, allowing homogenization of lots during harvest. Fruit color has been adopted as a marker in species such as *Campomanesia xanthocarpa*, *Myrtaceae* family. (Herzog et al., 2012), *Tibouchina granulosa*, *Melastomataceae* family (Lopes et al.,2005) and *Peschiera fuchsiaefolia*, *Apocynaceae* family (Martins et al.,2004), being an efficient method to determine the physiological maturity of seeds.

Considering the economic importance of tobacco production and the lack of information concerning physiological maturity of different varieties and varietal groups, we evaluated the possibility of using fruit appearance as an indicator of physiological maturity for two important commercial tobacco cultivars, CSC 444 (Virginia varietal group) and CSC 221 (Burley varietal group).We also studied the physiological and biochemical changes in their seeds in order to establish the optimal time for harvest. The objective was to identify possible biochemical markers for assessing the physiological quality of tobacco seeds before and after harvesting.

MATERIAL AND METHODS

Seeds of *N. tabacum* were obtained from two distinct CMT seed fields: one with plants of cultivar CSC 444 (Virginia) and the other with cultivar CSC 221 (Burley). Fruits were harvested at various maturity stages, based on their appearance. The fruits were classified as: green (G), green with dark apex (GD), partially dark (PD), totally dark (TD), and dry (D).

The experimental design was based on randomized blocks with five treatments (fruit ripening stages) and four replicates. At harvest time, each field was divided into four blocks to collect 500 fruits per stadium; these fruits were packed in nonwoven bags. Then, the fruits were submitted to a drying process, where they remained in a stationary dryer at a constant temperature of 35°C, until the seeds reached a water content of 70 mg/ml. To monitor the drying process, the seed water content was determined by oven drying at high temperature with two subsamples in an oven maintained at a temperature of 130-133 °C for 1 hour (Brasil, 2009). After drying, seeds were manually extracted and processed in 30-mesh sieves. Later, they were packed in waterproof containers and sent to the laboratory to proceed with the evaluations. In the laboratory, the seeds of each treatment were placed on a clear and smooth surface, and homogenized manually, stacked and divided in half using a ruler. One of the halves was disregarded, and the same was done with the other half, until a sample weighing 0.3g (Brasil, 2009).

The germination test was conducted with 200 seeds (four replicates of 50), for each stage of the fruits of the two cultivars, distributed over a blotting paper substrate moistened with a potassium nitrate (KNO_3) solution at a concentration of 2 mg/ml corresponding to 2.5 times the weight of the dried substrate placed in transparent plastic gerbox-type boxes (11 × 11 × 3.5 cm). Seeds were kept in a BOD germinator at a temperature of 20-30°C and a daily photoperiod of eight hours. The first germination was counted at day seven and the germination by count of normal seedlings was assessed at day 16. The results were expressed as percentages (Brasil, 2009).

The germination speed index (GSI) was determined along with the germination test, by a daily count of root protrusion. The expression proposed by Maguire (1962) was used for calculations.

Time to reach 50% germination (T_{50}): was calculated by the equation proposed by Guimarães (2000) for the time required for the occurrence of 50% germination:

$$T50 = [(G - G1)I / G2 - G1] + T \quad \text{(Equation 1)}$$

T50 = Time to reach 50% germination
G = Half of the maximum germination value
G_1 = Germination value equal or less than G;
G_2 = Germination value immediately above G;
I = Interval between counts;
T = Time for occurrence of G1

Cumulative average germination was calculated by a daily count of root protrusion for 16 days, and plots were made for each stage with the data.

Seedling emergence and emergence speed was measured in a greenhouse. There were four replicates of 50 seeds for each treatment. The seeds were sown in polystyrene trays of 200 cells using commercial substrate (Mecplant®). After sowing, the trays were maintained in "floating" system in a 5-cm water depth. The percentage of seedlings was evaluated at day 21.

Isozyme profiles were determined from two subsamples of dried were soaked in polyvinylpyrrolidone and liquid nitrogen, in porcelain crucibles. For each enzyme, 100 mg of macerated sample was placed in wells with 250 µL extraction buffer (0.2 M Tris HCl (hydrochloric acid), pH 8.0) and 1 mg/ml β-mercaptoethanol. They remained overnight and then were centrifuged at 14,000 rpm for 30 minutes at 4°C. An aliquot of 60 µL of the supernatant was removed and applied in 75 mg/ml and 45 mg/ml polyacrylamide gel (separating gel and concentrating gel, respectively). The electrophoretic run was subjected to a constant voltage of 120 V for five hours. After this period, the gels were read for the enzymes CAT - EC 1.11.6.1, EST - EC 3.1.1.1, IDH - EC 1.1.1.41, MDH - EC 1.1.1.37 and ADH - EC 1.1.1.1, using the methodology contained in Alfenas (2006).

For the heat-resistant protein analysis, tobacco seeds were soaked for 5 hours and macerated in a crucible in liquid nitrogen, adding extraction buffer at a ratio of 10:1 (buffer/sample). The samples were centrifuged at 14,000 rpm for 30 minutes at4°C. The supernatant was separated and incubated in an 85°C water bath for 15 minutes following centrifugation, as previously described. Then, the samples were placed in a water bath at 100°C and then run through electrophoresis. Following methodology contained in Alfenas (1998), electrophoretic run was performed for 12 h and subsequently gels were stained with 0.5 mg/ml Coomassie Blue solution and 100 mg/ml acetic acid solution for discoloration to visualize the bands.

For the extraction of endo-β-mannanase enzyme, 300 µL extraction buffer (0.1 M Hepes/0.5 M NaCl (sodium chloride) and ascorbic acid (5 mg ascorbic acid per ml of buffer), pH 8.0) were added to each well with 100 mg of powder of seed sample. The samples were then centrifuged for 30 minutes at 14,000 rpm and 2 µL of the supernatant was applied to gel containing 6 ml of LBG (locust bean gum), 0.24 g of agarose, and 24 ml of pH 5.0 buffer (1 M citric acid/0.4 M Na_2HPO_4(sodium hydrogen phosphate)and 2 M H_2O (hydrogen oxide)). The aliquots were applied to 2 mm holes made in the gel with the aid of an awl. The gel was incubated for 21 h and revealed according to the methodology proposed

by Silva *et al*. (2004). The endo-β-mannanase enzyme activity was calculated according to Downie et al. (1994).

The data were submitted to analysis of variance and means were compared by the Scott-Knott test at a 0.05 significance level. The results from the enzymatic profiles were read based on visual analysis of electrophoresis gels, considering the presence/absence and the intensity of each isozyme electrophoretic band in each system.

In a second experiment, six lots of CSC 444 were used to identify possible biochemical markers in the physiological quality of commercial tobacco seeds. In the characterization of these lots, germination and emergence tests were carried out and the germination and emergence speed indices were calculated as previously described.

The seed of those lots were run through electrophoretic analysis of CAT, EST, MDH, ADH, and heat-resistant proteins, according to the methodology described above. The experiment was conducted in a completely randomized design with four replicates of 50 seeds. Statistical analysis was performed with SISVAR and comparison of means was performed by the Scott-Knott test at a 0.05 significance level.

RESULTS

Regardless of the fruit harvest stage and cultivar, the first evidence of the germination process (root protrusion) occurred on the fourth day after planting (Figure 1). This analysis was made possible by monitoring germination.

In cultivar CSC 444, the maximum values of cumulative germination were identified in the partially dark, totally dark, and dry stages. In cultivar CSC 221, cumulative germination was low in the early stages of ripeness (green and green with dry apex), and maximum stability, uniformity, and germination were obtained for seeds in the dry stage.

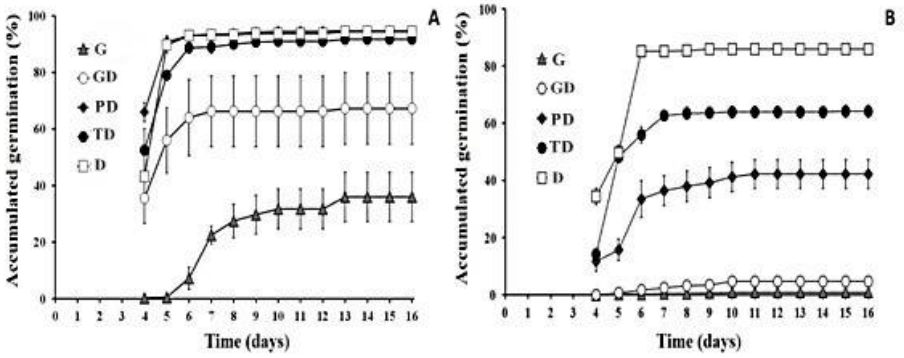

Figure 1. Cumulative germination of tobacco seeds of cultivars CSC 444 (Virginia varietal group) (A) and CSC 221 (Burley varietal group) (B), from fruits harvested at different maturity stages: green (G), green with dark apex (GD), partially dark (PD), totally dark (TD), and dry (D).

For both cultivars, seeds from fruits in the dry stage showed rapid and uniform germination, so that on the fifth day they no longer had significant changes in the process. When the fruits were harvested in partially dark and totally dark stages, the stabilization of seed germination occurred on the ninth day. In CSC 444, the cumulative germination was

similar in the partially dark, totally dark, and dry stages, whereas in CSC 221 these stages had lower germination than in the dry stage.

In seeds from fruits in green and green with dark apex stages, germination was lower and slower compared to the seeds of the other stages, with stabilization in the eighth and tenth day for the respective stages. Cultivar CSC 444 presented a significant difference in all the variables, whereas cultivar CSC 221 presented no significant effect on the time required for the occurrence of 50% germination (Table 1).

Table 1. First count (FC), germination (GER%), time to reach 50% germination (T_{50}), emergence (EM%) and germination speed index (GSI) averages of tobacco seeds of CSC 444 virginia varietal group and CSC 221 burley varietal group, from fruits harvested at different maturity stages: green (G), green with dark apex (GD), partially dark (PD), totally dark (TD) and dry (D).

CULTIVAR – CSC 444					
Maturation stages	FC	GER	T_{50}	EM	GSI
		----------(%)----------			(index)
Green	10 C	35 C	7.75 C	1 E	4.7 C
Green with dark apex	63 B	67 B	5.00 B	64 D	15.0 B
Partially dark	92 A	94 A	4.25 A	96 A	22.0 A
Totally dark	87 A	92 A	4.75 B	82 C	20.5 A
Dry	90 A	94 A	5.00 B	85 B	20.7 A
CV(%)	13.67	13.36	7.24	3.00	18.17
CULTIVAR – CSC 221					
Maturation stages	FC	GER	T_{50}	EM	GSI
		----------(%)----------			(index)
Green	0 D	1 D	5.37 A	5 C	0.1 D
Green with dark apex	2 D	4 D	7.85 A	12 C	0.55 D
Partially dark	33 C	40 C	5.93 A	62 B	7.6 C
Totally dark	55 B	63 B	5.21 A	65 B	12.7 B
Dry	79 A	86 A	5.04 A	82 A	17.7 A
CV(%)	14.12	11.78	12.54	15.63	14.87

Means followed by the same letter in the columns do not differ by the Scott-Knott test at 5% probability.

In a comparison of the average emergence values presented in Table 1, we noted that the greatest effect was obtained in seeds harvested from fruits in the partially dark stage, and from this stage on there was a tendency to decrease. The T_{50}results also showed high vigor for seeds in that stage when compared to the other stages. In the T_{50} test, seeds in the green stage present low vigor, seeds in the green with dark apex, totally dark, and dry stages had intermediate vigor, and seeds in the partially dark stage had high vigor.

CSC 221 had a germination advance and more pronounced vigor throughout physiological maturity. Significant quality increases were observed from the partially dark to the dry stage, the latter showing higher levels.

Unlike CSC 444, the germination of seeds of CSC 221 was above the minimum standard only when extracted from dried fruits. The remaining stages showed viable seeds but with lower germination and vigor, and thus lower physiological performance. Therefore, according to the first count, germination, germination speed index, and emergence, the dry stage is ideal for harvesting CSC 221.

In CSC 444, the presence of a band in the green stage for catalase activity was not observed (Figure 2A). This presence, as well as increased activity, occurred in the green with dark apex stage, which also presented an increase in seed vigor assessed by accumulated germination. No significant differences were observed in CAT activity for the other stages.

For CSC 221, fruits harvested in the green and green with dark apex stages showed low CAT activity, and germination and vigor were practically absent in these stages (Figure 2B).

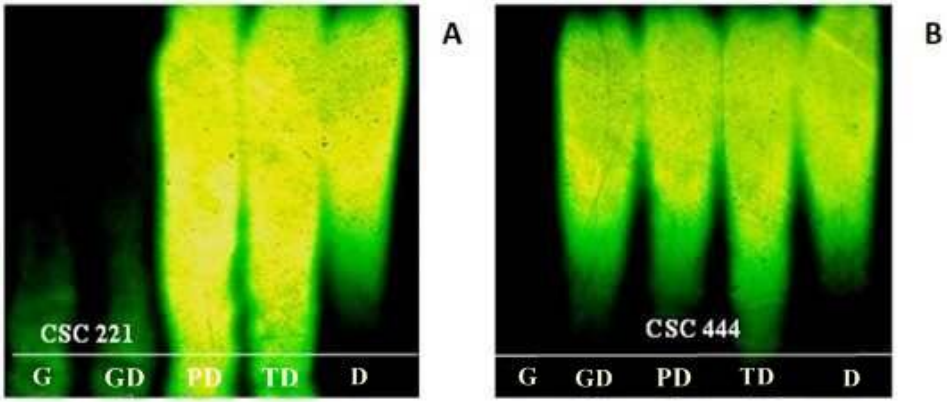

Figure 2. Enzymatic profile of catalase (CAT), extracted from tobacco seeds of CSC 444 (A) and CSC 221 (B), from fruits harvested at different maturity stages: green (G), green with dark apex (GD), partially dark (PD), totally dark (TD), and dry (D).

Differences in esterase activity were observed in both cultivars for seeds in different stages (Figure 3A and B). For both CSC 444 and CSC 221, activity of esterase was found in fruit seeds with green coloring (green and green with dark apex), but with less intensity. With advancement in fruit ripening, there was a greater band intensity.

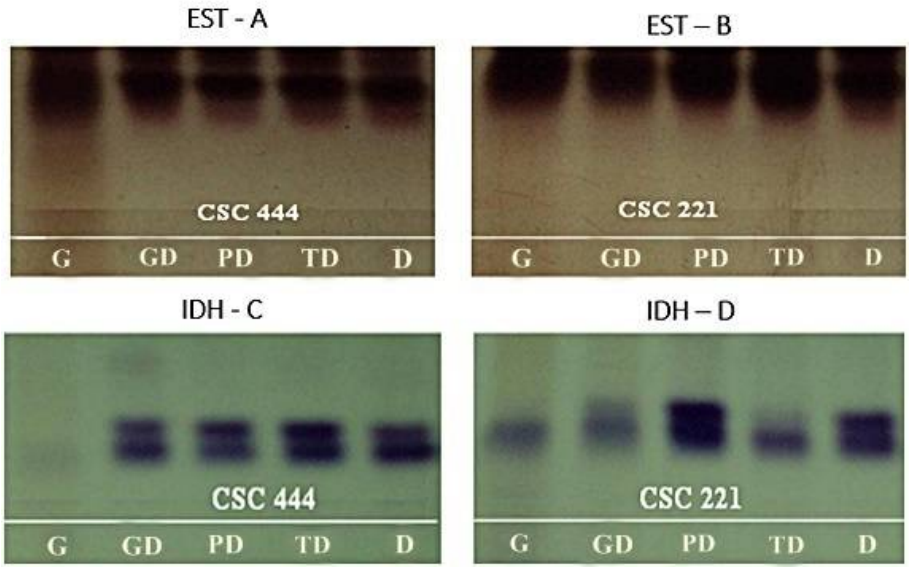

Figure 3. Enzymatic profile of esterase (EST) and isocitrate dehydrogenase (IDH) enzymes, extracted from tobacco seeds of CSC 444 (A and C) and CSC 221 (B and D), from fruits harvested at different maturity stages: green (G), green with dark apex (GD), partially dark (PD), totally dark (TD), and dry (D).

For CSC 221, similar to the catalase enzyme results, we observed increasing activity of esterase in fruits that had a green with dark apex appearance, with subsequent reduction in dry fruits. During the change from dark green to partially dark stages, there was an increase in the enzymatic profile of the IDH enzyme for both cultivars (Figure 3C);this is related to the moment that the cultivars reach physiological maturity, given the higher percentages of germination and vigor (Tables 1 and 2). This enzyme is involved in the aerobic respiratory process and functions in the Krebs cycle (Taiz and Zeiger, 2004).

CSC 221 had the same patterns of catalase and esterase activities; there was increasing activity in the green with dark apex stage, with subsequent reduction in the dry stage (Figure 3D).

MDH had a similar catalase pattern in both cultivars. In CSC 444, less intensity was observed in the green stage, and there was an increase in activity with maturation in the green with dark apex stage (Figure 4A). In CSC 221, fruits harvested in the green and green with dark apex stages had low MDH activity, and an increase was noted with increasing maturity (Figure 4B).There was more ADH activity in seeds from fruits harvested in the partially dark stage in cultivar CSC 444 and in the dry stage in cultivar CSC 221 (Figure 4C and D).

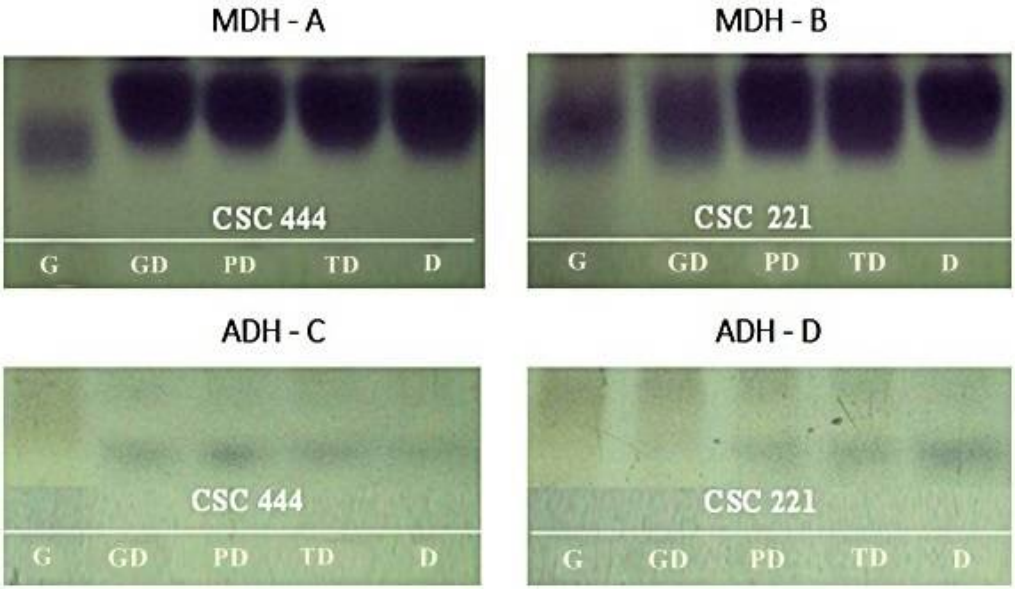

Figure 4. Enzymatic profile of malate dehydrogenase (MDH) and alcohol dehydrogenase (ADH) enzymes, extracted from tobacco seeds of CSC 444 (A and C) and CSC 221 (B and D), from fruits harvested in different stages of maturity: green (G), green with dark apex (GD), partially dark (PD), totally dark (TD), and dry (D).

Lower expression of heat-resistant proteins was observed in both cultivars (Figure 5) in seeds extracted from fruits in the green stage, and increased expression in fruits in the green with dark apex stage.

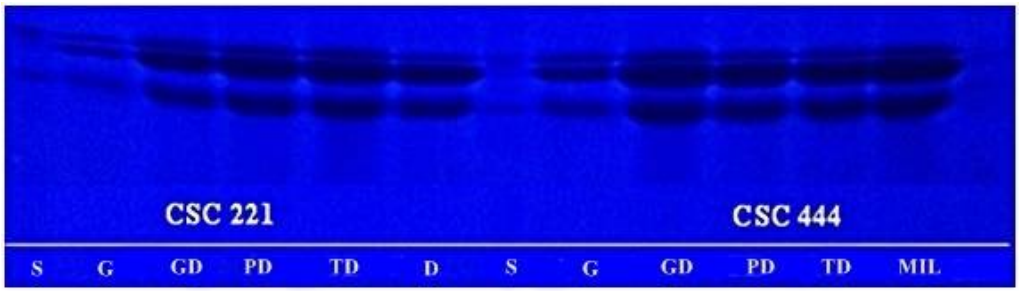

Figure 5. Electrophoretic profile of heat-resistant proteins in tobacco seeds extracted from Burley and Virginia varietal groups, from fruits harvested at different maturity stages: standard (S), green (G), green with dark apex (GD), partially dark (PD), totally dark (TD), and dry (D).

There was activity of endo-β-mannanase in seeds of fruits at all ripening stages, and seeds from fruits harvested at the green stage showed lower activity (Figure 6).In CSC 444, the increased activity of these enzymes appeared from the partially dark stage to the other stages (Figure 6A).In CSC 221, this increase was also observed in seeds from partially dark fruits. However, there was a reduction of expression in the following stage (totally dark), with a further increase in the dry stage (Figure 6B).

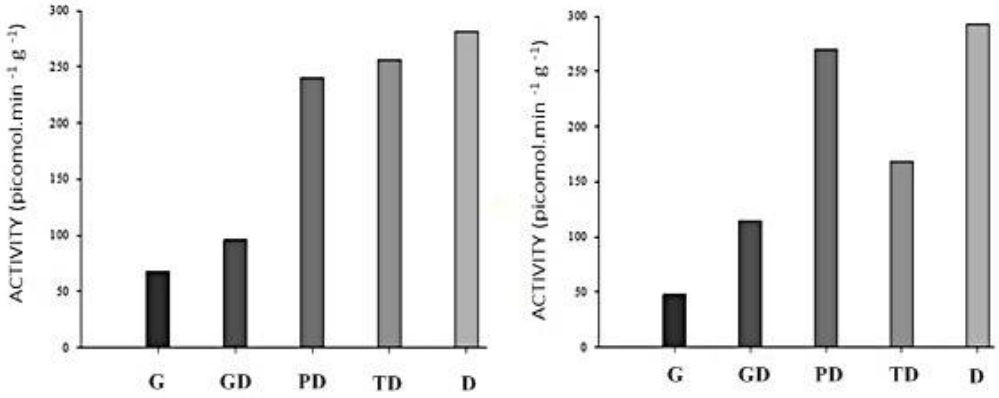

Figure 6. Endo-β-mannanase enzyme activity of CSC 444 (A) and CSC 221 (B) from fruits harvested at different maturity stages: green (G), green with dark apex (GD), partially dark (PD), totally dark (TD), and dry (D).

The germination test of lot 2 in the second experiment gave an average higher than the other lots. Lots 3, 4, and 5, although they had lower germination than lot 2, were considered high-quality lots (Table 2).

In the germination speed index, lots 2 and 4 were classified as high-quality, and lots 3, 5, and 6 were classified as intermediate quality. When we assessed emergence, lots 2 and 4 had higher vigor and lots 3, 5, and 6 had an intermediate effect compared to the other lots. Similar results were also observed in the assessment of the emergence speed index.

Table 2. Germination percentage (GER%), germination speed index (GSI), emergency percentage (EP%) and emergence speed index (ESI) of tobacco seeds of six lots of CSC 444 cultivar (virginia varietal group).

LOT	GER (%)	GSI	EP (%)	ESI
1	03 D	00.25 C	10 C	0.50 C
2	95 A	12.45 A	93 A	6.38 A
3	87 B	10.14 B	72 B	4.89 B
4	87 B	11.80 A	97 A	6.44 A
5	89 B	10.39 B	74 B	4.80 B
6	79 C	09.45 B	76 B	5.07 B
VC(%)	6.30	10.17	10.71	11.39

Means followed by the same letter in the column do not differ by the Scott-Knott test at 5% probability

When analyzing CAT activity (Figure 7A), increased expression was observed in tobacco seeds from lots 2 and 6. These same batches were considered to have low physiological quality in the germination test compared to the other lots (Table 1).

In the EST enzymatic pattern analysis (Figure 7B), greater expression of this enzyme was observed in seed from lots 1 and 6, similar to what occurred with CAT (Figure 7A), in which most enzyme activity was observed in lots with lower physiological quality. The ADH enzymatic profile (Figure 7C) did not show variation in the intensity of bands among all treatments. When we examined MDH enzyme expression, lower intensity of the bands was observed in lot 2 (Figure 7D).

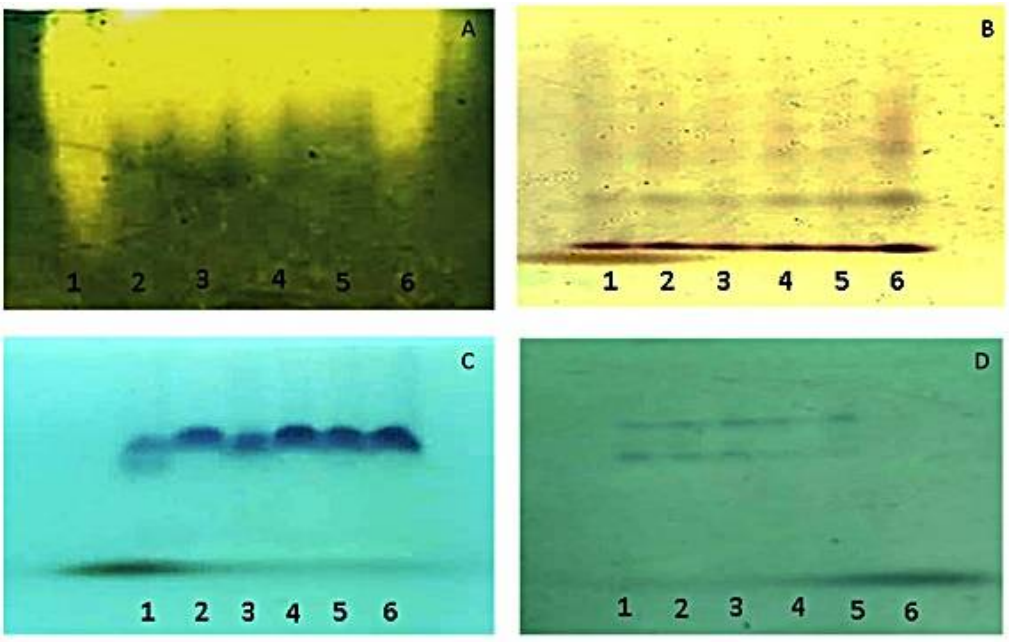

Figure 7. Enzymatic profile of catalase (CAT) – A, esterase (EST) – B, alcohol dehydrogenase (ADH) – C, and malate dehydrogenase (MDH) – D, extracted from six lots of tobacco seeds (lots 1, 2, 3, 4, 5 and 6) of CSC 444.

Figure 8 shows the expression profile of heat-resistant proteins. The band intensity for this protein was similar in all lots.

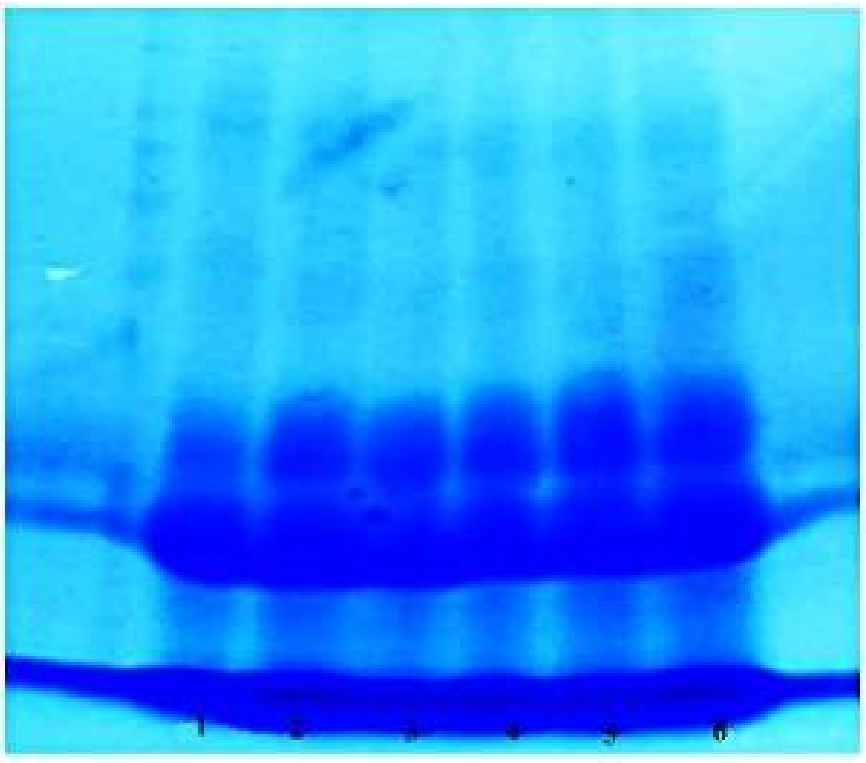

Figure 8. Electrophoretic profile of heat-resistant proteins extracted from six lots of tobacco seeds (lots 1- 6) of CSC 444.

DISCUSSION

For cumulative germination of tobacco seeds of cultivars CSC 444 and CSC 221 from fruits harvested at different maturity stages, regardless of the fruit harvest stage and cultivar, the first evidence of the germination process (root protrusion) was apparent on the fourth day after planting. This analysis was made possible by monitoring germination. Ikeda et al. (2013) indicated that accumulated germination expresses germination behavior of a variety over time, which may indicate differences in stability and uniformity that would be observed with seed germination. Germination and emergence uniformity is essential for tobacco seed production. According to Hartle et al. (2002), unevenness in the emergence of tobacco seedlings can reduce the percentage of useful transplants, and the growth of seedlings that emerge later will be affected by the shading of the closest developed seedlings.

Regardless of cultivar, seeds from fruits harvested in green and green with dark apex stages had a lower physiological quality. This fact is related to their immaturity stage. During maturation, from the partially dark stage on, there was a progressive increase in germination and seed vigor.

In the initial stages, there are constant physical, physiological, and biochemical changes linked to seed formation, which justifies the low germination and vigor values.

Therefore, it is not recommended to harvest these fruits. It can be inferred that early harvest from these stages can yield lots with numerous unripe seeds, leading to a reduction in quality. Among these changes, there is endogenous accumulation of abscisic acid, present in greater amounts in the immature seed, leading to the appearance of primary dormancy and preventing seed germination (Kerbauy, 2008). within their study of the physiological maturity of pepper seeds, Queiroz et al. (2011) and Ricci et al. (2013) have also shown that low-quality lots were formed when fruits were harvested too early (green stage). According to Amaral et al. (2000), in quality evaluation of *Bixa orellana* seed, immature seeds harvested in the early stages did not germinate, as was also found by Passam et al. (2010) in the early ripening stages of eggplants. For CSC 444, in the partially dark stage, germination was higher than the minimum standard established for the marketing of tobacco seeds (≥ 80%). Because they have similar percentages of germination, differences between the partially dark, totally dark, and dry stages were not relevant. Probably, this fact is associated with the sensitivity of these tests, since, for Reis et al. (2012), lots with similar germination but with different vigor levels are not differentiated in the germination test. Differences were noted between the first count and the germination speed index.

Regarding the emergence average values, we noted that the greatest values were obtained in seeds harvested from fruits in the partially dark stage, and from this stage on there was a decreasing tendency. Results obtained for T_{50} also showed high vigor for seeds in that stage when compared to the other stages. Carvalho and Novembre (2011) have reported low efficiency in the germination and first count tests in quality distinction between different lots of tobacco seeds compared to the emergence test. In their study, the emergence test allowed the classification of lots at different vigor levels.

Unlike CSC 444, germination of seeds of CSC 221 was above the minimum standard only when extracted from dried fruits. The remaining stages showed viable seeds but with lower germination and vigor, and thus lower physiological performance. Therefore, according to the first count, germination, germination speed index, and emergence, the dry stage is ideal for harvest of CSC 221 seeds.

Some care must be taken to avoid delay in the harvest of CSC 221 fruits, as possible seed losses may occur with natural dehiscence of the capsules, because they stay longer in the field to reach the dry stage. Natural dehiscence can reduce production and lead to exposure of seeds to fungi and insects, which will lead to further deterioration of seeds in the field and reduction of physiological and sanitary quality. More attention is needed at the harvest of these fruits when the capsules reach the ideal time for harvest (dry stage).

For both tobacco cultivars, we identified the ideal time to harvest by their appearance, obtaining seeds with higher physiological quality. However, this stage may vary depending on the cultivar. These results are in accordance with Demir and Samit (2001), who reported that the staining of tomato fruits was more efficient for identifying the physiological maturation of the dry matter content.

The catalase enzyme profile resembles the results of physiological quality, in which both cultivars presented low quality in early stages because of the percentage of seed in formation (immature) and enzymatic apparatus still in development. These results confirm those found by Albuquerque et al. (2009) when studying *Capsicum annuum* seeds, who also found low CAT activity in immature seeds.

The activity of this enzyme is related to the hydrogen peroxide decomposition formed by SOD (superoxide dismutase) in the cells, acting as a second line of defense

(Mallick and Mohn, 2000). Reduction in CAT activity can make the seed more sensitive to the effects of free radicals on unsaturated membrane fatty acids, compromising its strength (Albuquerque et al., 2009).

For CSC 221, similar to the results for the catalase enzyme, we observed increasing activity of esterase in fruits that had a green with dark apex appearance, with a subsequent reduction in dry fruits. According to Santos et al. (2004), changes in esterase enzyme patterns are correlated with deteriorative events, since this enzyme is related to ester hydrolysis reactions, acting on lipid metabolism.

MDH showed a similar catalase pattern in both cultivars. This enzyme acts as a catalyst for the conversion reaction of malate into oxaloacetate for the production of NADH during the Krebs cycle. According to Bray et al. (2000), the reduction of its activity promotes disruption of mitochondrial membranes, impairing the production of ATP and oxygen absorption.

There was more ADH activity in seeds from fruits harvested in the partially dark stage in cultivar CSC 444 and in the dry stage in cultivar CSC 221. This similarity in the results of ADH profile and the results of seed quality in these cultivars allows us to infer that this enzyme can be assessed for predicting the quality of tobacco seeds, since the enzymatic profile coincided with the physiological potential of seeds evaluated by accumulated germination, first count, germination, seedling emergence, and germination speed index.

These results confirm what was found by Vidigal et al. (2009) in their study with *Capsicum annuum* seed and by Brandão Junior et al. (2002) in their study with *Coffea arabica* seed, who observed a higher correlation of ADH in determining the physiological quality of seeds extracted from fruits at different stages of maturation. The activity of this enzyme occurs in anaerobic respiration, being responsible for the metabolism of ethanol into acetaldehyde (Veiga et al., 2010). Acetaldehyde accelerates the deterioration of seeds (Zhang et al., 1994); therefore, with increased ADH activity, seeds are more protected against the harmful action of this compound and thus have more vigor.

There was lower expression of heat-resistant proteins in both cultivars in seeds extracted from fruits in the green stage, and increased expression is seen in fruits in the green with dark apex stage. Thus, seeds from fruits harvested from the green with dark apex stage on may have greater tolerance to desiccation. This class of proteins is responsible for the protection and stabilization of the cellular membrane, retaining water and preventing crystallization of molecules due to dehydration (Taiz and Zeiger, 2004). According to a study by Faria et al. (2004) with *Zea mays* seeds before drying, there were less heat-resistant proteins in the early stages of milk line (ML) to ML-3, when they showed inferior quality. However, after drying, they observed that all maturation stages showed the same physiological quality, with no protein expression.

There was activity of endo-β-mannanase in seeds of fruits in all ripening stages; seeds from fruits harvested at the green stage showed lower activity. In CSC 444, the endo-β-mannanase standard band corresponds with the standards of CAT, EST, IDH, and ADH enzymes, with the heat-resistant proteins, and with the physiological potential of seeds. Thus, these analyses made it possible to infer that tobacco fruits can be harvested from the partially dark stage on, because seeds have higher quality. Veiga et al. (2007), in their study with *Coffea*, also found association of endo-β-mannanase enzyme activity with the quality of seeds, so that the highest enzyme activity correlated with the best quality of seeds

extracted from fruits harvested at later stages, compared to fruits in early maturation. However, these results differ from those reported by Dahal et al. (1997), who found no relation between the activity of endo-β-mannanase and germination in seeds of *Lycopersicon esculentum*.

The endo-β-mannanase enzyme is considered essential to the seed germination process, being directly related to the endosperm softening process (Kucera et al., 2005 and Silva et al., 2004). In tobacco, this softening is essential to the process of seed germination. According to Manz et al. (2005), in tobacco seeds, in addition to the seed coat, the embryo is coated with three or five layers of endosperm cells. These authors consider that these characteristics give two obstacles to the germination of tobacco seeds. The first is breaking the seed coat and the second is breaking the endosperm. Thus, it is important to evaluate the endo-β-mannanase in these seeds.

We found fruit appearance to be an indicator of fruit maturity and tobacco seed quality. The ideal stage of fruit maturation varies according to the cultivar. Partially dark CSC 444 fruits and dried CSC 221 fruits provide better quality seeds. The ADH enzyme indicates the optimal stage to harvest fruits of cultivars CSC 444 and CSC 221.

For CAT activity increased expression was observed in tobacco seeds from lots 2 and 6. These same batches were considered to have low physiological quality in the germination test compared to the other lots. Taveira et al. (2012), who studied *Coffea* seeds, also observed higher catalase enzyme expression in seeds with lower physiological performance. However, Cruz et al. (2013), who analyzed *Crambe abyssinica Hochst*seeds for vigor differences, found the opposite result, in which a reduction of physiological quality was related to low expression of catalase.

Regarding MDH enzyme expression assessment, a lower intensity of the bands was observed in lot 2. Similar results were found in the evaluation of the MDH activity in *Glycine max* seeds stored in uncontrolled conditions, as well as a reduction in the expression of the enzymatic system when the seeds had less vigor and low germination percentage (Carvalho et al., 2014). The increase in MDH expression in rice seeds exposed at 35°C may occur because of increased seed respiration. Therefore, one can conclude that MDH can be used to identify the beginning of the deterioration process in *Oryza sativa* seeds (Marini et al., 2012). Thus, catalase and esterase enzymes could be considered biochemical indicators to assess the stage of deterioration of lots of tobacco seeds.

CONCLUSION

The appearance of fruits is an indicator of fruit maturity and quality in tobacco seeds. The alcohol dehydrogenase enzyme indicates the optimum stage to harvest fruits. The catalase and esterase enzymes are biochemical indicators that can assess the deterioration of tobacco seed lots.

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