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Proline: use as an indicator of temperature stress in bean seeds

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ABSTRACT *- Temperature stress can lead to several metabolic alterations along the entire plant cycle, including germination. To verify the effect of high or low temperatures, constant or in alternate cycles, 10 bean genotypes (eight cultivars and two landraces) were sown to germinate in trials for proline quantification, which were conducted in a climatic chamber under five thermal treatments: T1 - suboptimal temperature (96h at 8 ºC); T2 - cold shock (48h at 18 ºC followed by 48h at 8 ºC); T3 - optimal temperature (96h at 18 ºC); T4 - heat shock (48h at 18 ºC followed by 48h at 37 ºC) and T5 - supra optimal temperature (96h at 37 ºC). Proline was quantified at 520 nm in a spectrophotometer. There were responses to different environments for each genotype. The assessment of proline is recommended in germinating seeds as a means to discover stress temperature responsive bean lines, as for example 'Guarumbé'.*

Key words: *Phaseolus vulgaris*, heat shock, cold shock, germination, amino acids.

INTRODUCTION

In completely developed tissues there are some biochemical responses to environmental changes in temperature, water deficit, or flooding and salinity. The induced metabolism alterations could be assessed by measuring alterations in the enzyme activities, increments in amino acid, e.g. proline concentrations (Marur et al. 1994, Arora and Saradhi 1995), and specific stress proteins, e.g. heat shock proteins - HSP (Vierling 1991) or water deficit (Andrade et al. 1995) and salinity proteins (Lutts et al. 1996a).

Plant adaptation to a non-favourable environment is based on a strong need for survival. Crops of temperate and tropical origin are affected when exposed to low temperatures. Cold injure is a physiological disorder that is developed in some tropical and subtropical plants when they are exposed to temperatures under 10 to 12 ºC, though not to freezing temperatures (Sabehat et al. 1996). Rab and Salveit (1996) reported germination and growth reduction and loss of viability as symptoms of temperature injure.

Temperature has an important yield-limiting factor in Phaseolae, especially due to the influence on germination and emergency (Zaiter et al. 1994). A relation between germination and growth rate had been documented in bean, e.g., seeds that exhibited a slow germination will probably have reduced growth. In view of this characteristic of the Phaseolae tribe,

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particularly in *Phaseolus*, several studies (Braak and Koistra 1975, Hardwick and Andrews 1980, Zaiter et al. 1994) have been realized to develop low temperature-tolerant breeding lines for cold regions or supra optima temperature-tolerant lines for high temperature regions. This condition is particularly important in the tropical regions that make up most part of the Brazilian territory, where temperatures at the soil surface do not seldom exceed 45-50 °C. Kasalu et al. (1993) observed that soil temperatures over 45 °C inhibited germination and seedling emergency in sorghum. The optimal temperature range was between 21-35 °C, and the lethal range would vary from 40 to 48 °C.

Marur et al. (1994) argued that plant tissues showed several alterations in the metabolic pathway as a kind of response to the water deficit. These alterations could occur at three levels: i) perturbation of the metabolic pathway leading to an increase or loss of metabolites; ii) alterations in enzymatic activities; iii) and changes in the protein patterns. Proteins play a fundamental role in the plant response to temperature.

Ferguson et al. (1990) reported that high temperatures could directly or indirectly damage plant proteins by enzyme inactivation, by alteration in peptide configuration, or by the loss of organisation in membrane complexes. In this knowledge area, important progress has been achieved with insights into molecular responses to high temperature or heat shock (Vierling 1991). Several studies have investigated electrophoretical patterns, which are a useful tool in other research areas such as environmental stress response (Marmiroli et al. 1989, Siegel 1993).

Solute accumulation is the response of an organism facing environmental stress (Trotel et al. 1996, Hoai et al. 2003, Rizhsky et al. 2004), initially used as osmotic regulator and membrane and enzyme protector, and then as carbonic backbone and amine reservoir to *de novo* synthesis and growth restarting when the stress is over (Al-Karaki et al. 1996).

The ability of higher plants to use proline could confer some advantage to populations that had undergone water deficit for the high energetic and nutritional value of this nitrogen-containing compound (Trotel et al. 1996). Proline seems to be related to the maintenance of protein stability for maturation or for elongation (Andrade et al. 1995). Accumulation could be related to proline synthesis or degradation (Sudhakar et al. 1993), as with the inhibition of protein synthesis, and in the case of beans apparently related to the first mechanisms (Andrade et al. 1995).

Proline synthesis could be related to photoinduction and/or photoinactivation of proline catabolism-associated enzymes. Stewart et al. (1966), Bates et al. (1973), and Stewart and Larher (1980) showed the essential role of proline as osmotical solute during stress conditions. However, the increase in proline content indicated resistance or tolerance to water deficit, serving as a parameter for the selection of plant material with high resistance. On the other hand, Maggio et al. (2002) showed that proline-accumulating materials were susceptible to this kind of stress. The increase of this amino acid will allow the maintenance of the osmotic equilibrium during growth even when subjected to water deficit, acting as nitrogen and carbon source after the stress period (Sudhakar et al. 1993, Al-Karaki et al. 1996, Hoai et al. 2003).

As HSP, proline seems to be linked to several kinds of stresses as an alternative pathway to minimise their effects and as an osmoprotector of molecules and membranes, forming walls of hydration over the phospholipids and reducing the action of free radicals, linked to these and producing chemically stable molecules. While proteins and other macromolecules are dehydrated in their native state, some integrity can be maintained if the water stays close to these molecules preventing the deformation or fragmentation of the same (Alia and Saradhi 1991, van Rensburg et al. 1993, Lutts et al. 1996a, b).

The temperature effect during germination can be looked at differently. In this study, the biochemical pathway was observed under temperature stress. Evaluations compared two different cultivars from two fields and ten cultivars from the same field, considering the increase of proline during germination.

MATERIAL AND METHODS

The following cultivars of snap beans (*Phaseolus vulgaris* L.) were used: 'Rosinha G-2', 'IAC-Carioca-80SH', 'Vermelho 2157', 'IAPAR 57', 'Rudá', 'Aporé', 'Campeão-1', 'IAC-Carioca-Akytã' and the landraces 'Iratim' and 'Guarumbé', all multiplied on a field in Rio Claro, State of São Paulo at UNESP. All materials were obtained from the germplasm banks of Cenargen, Embrapa (Brasília, Federal District), IAC (Campinas, State of São Paulo) and IAPAR (Londrina, State of Paraná). Harvested seeds were wrapped in kraft paper bags, purged with phosphine, and stored in a temperature-controlled room at 25 °C.

A thermo gradient block with ten temperatures ranging from 45 to 8 ºC was used for germination. The thermo block was built based on a model of Labouriau and Agudo (1987). At least thirty seeds were scattered on propylene trays and covered with two sheets of germination paper moistened with distilled water. Each tray was inserted into a capped trial tube that was put into the cell of the thermo block, each temperature in five replications. The daily counts considered seeds with curved primary roots as germinated (Labouriau and Agudo 1987).

Germinability is the final percentage (x) of germination after eight days, transformed by the formula $arcsine\sqrt{x/100}$. Germination velocity (V) was determined by the expression: V=1/t, where t is the mean time for germination $\sum ni.ti/\sum ni$, being ni = number of germinated seeds in ti = time (Labouriau and Agudo 1987).

Germination trials for proline quantification were conducted in a climatic chamber under five thermal treatments: T1 - suboptimal temperature (96h at 8 ºC); T2 - cold shock (48h at 18 ºC followed by 48h at 8 ºC); T3 - optimal temperature (96h at 18 ºC); T4 - heat shock (48h at 18 ºC followed by 48h at 37 ºC); and T5 - supra optimal temperature (96h at 37 ºC). Proline was initially quantified after 96h, and in a second experiment, every 24h until 96h of thermal treatment. After that, they were taken out of the climatic chamber, ground in 3% sulphosalicylic acid and filtered through Whatman n.1 paper. This extract was used to quantify proline according to Bates et al. (1973) with some modifications. A total of 2 mL of the extract was added to 2 mL of acid ninhidrin and 2 mL of glacial acetic acid. The mixture was maintained at 100 ºC during 1h, after that the reaction was stopped in ice bath. Absorbance was measured spectrophotometrically at 520 nm. A calibration curve was established using four replicates per dose, which range from zero to 80 µg mL^{-1}, spaced at 5 µg mL^{-1} intervals. Samples with values over 80 µg mL^{-1} were diluted in glacial acetic acid and measured. Thereafter, data were converted to the original sample volume.

The germinability, germination velocity, and proline concentration were analysed by variance analysis and mean comparisons by Tukey's test at $P < 0.05$.

RESULTS

Seeds of 'IAC-Carioca-80SH' had the narrowest range of germination temperature (Figure 1) with no proline increase response but a decrease in cold treatments, which occurred either continuous (T1) or in shocking periods (T2 and T4 - Table 1). In 'Rosinha G2' seeds, there was an increase in proline production at all temperatures except for continuous 18 ºC (T3 - Table 1). This increase in proline concentrations was related to the need of amino groups to *de novo* synthesis of proteins, after the stress. This could be the explanation for the proline accumulation response in 'Guarumbé' and 'Iratim', where the temperature increase enhanced the proline content.

'Guarumbé' showed the highest proline yield of all cultivars (Table 1). For continuous heat (T5), there was an overproduction that was at least twice the amount of the heat-shocked seeds (T4). 'IAPAR 57' and 'IAC-Carioca-Akytã' did not respond to continuous heat (T5) and 'Rudá' to no temperature it was exposed to (Table 1). Both 'IAC-Carioca-Akytã' and 'Rudá germinated in a broad range of temperatures (Figure 1). 'Vermelho 2157' increased the amount of proline under either continuous cold (T1) or heat shock exposure (T4), and 'Campeão-1' showed a singular response under heat shock only, although germination dropped at 30 ºC (Figure 1). 'Aporé' increased the proline amount at temperatures of 18 ºC or 37 ºC or during the heat shock (T4 - Table 1).

However, the amount of proline during the whole period was not homogeneous (Table 2). For most of the cultivars there were no differences at the end of the first 24h, except for 'Guarumbé'. This cultivar had a high amount of proline after 24h of continuous cold (T1), with lower values after 96h under this treatment and a rise in proline 72h after the beginning of the experiment and 24h after the shock treatments (T2 and T4). When exposed to continuous heat, it reached the highest amount of proline found, however it failed to germinate at this temperature (Figure 1). 'Iratim' presented a similar pattern, but its proline production was much lower, although it was the second material in the total mean of proline accumulation and did not produce germinated seeds at 37 ºC, either. 'Vermelho-2157' increased the proline concentration after the start of heat shock (T4) at 37 ºC, decreasing after that, but when exposed to continuous heat (T5) the amount of proline dropped. 'Aporé' responded with a reduction after cold shock (T2) and a rise after heat shock or after 96h of 8 ºC or 18 ºC exposure, though not after exposure to 37 ºC.

A comparison of the results shown in Tables 1 and 2 exhibited two different kinds of response. The mean results for each cultivar/landrace could induce a wrong conclusion (Table 1). 'Iratin', for instance, appeared as a non-responsive line to cold temperatures (Table 1, T1). After 24h of shock temperatures, e.g. 72h, the same material again showed an increment in proline concentration (Table 2, T2). However, only the heat shock treatment (T4) was equal to the high temperature treatment (T5) in proline amounts when mean results were considered. The same phenomenon occurred with other cultivars, showing that 24h after changing the temperature, that is, 72h after the beginning of the experiments, there was an increase in proline synthesis responding to the shock temperature. Consequently, this is a very good moment to discriminate resistant materials such as 'Guarumbé' and 'Iratin'.

DISCUSSION

Amine-rich compounds are accumulated during cold shock response by the plant, and could be used after the

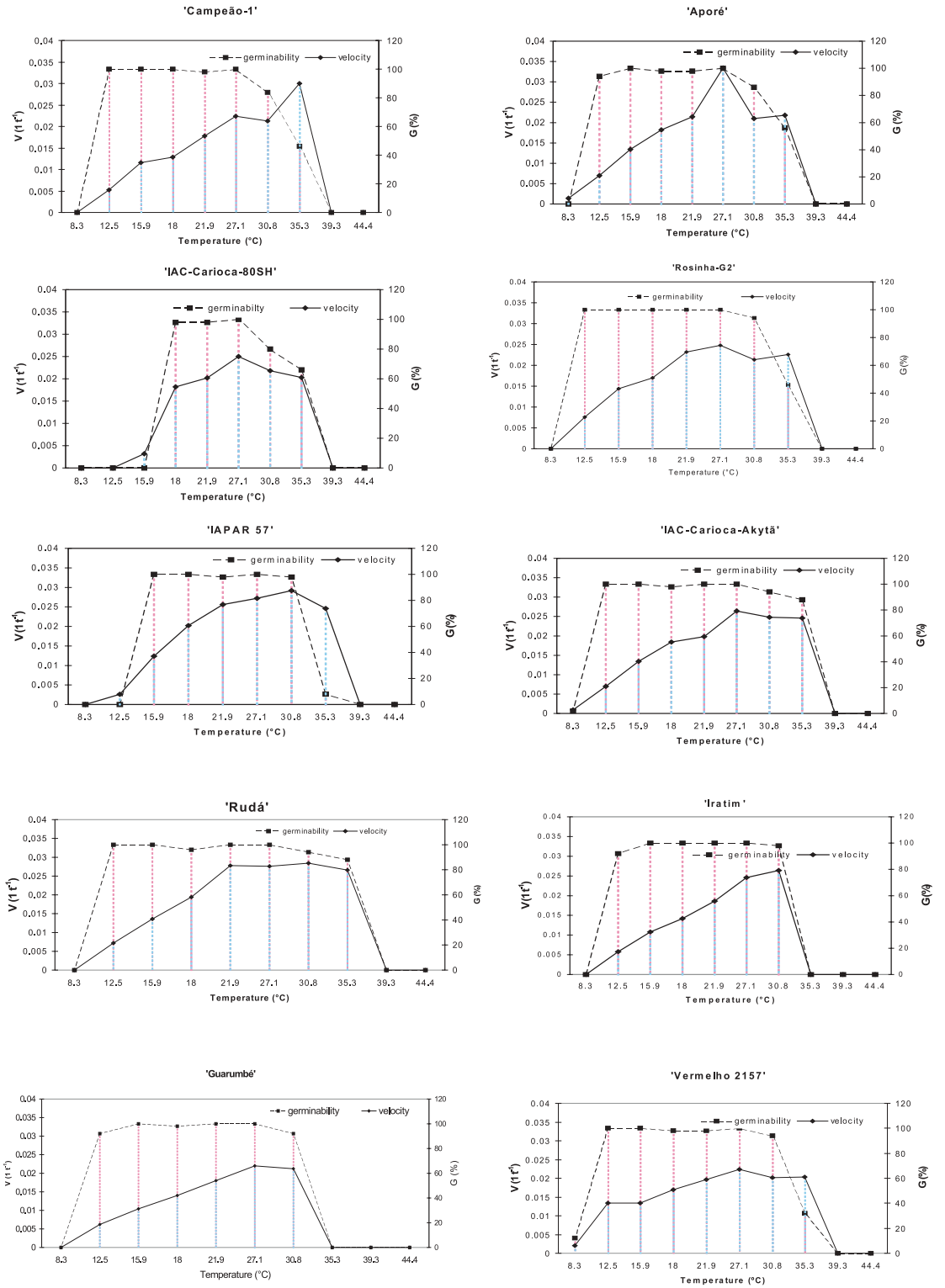

Figure 1. Germinability and primary root extrusion velocity of ten cultivars of *Phaseolus vulgaris*

shock as amine reservoirs or carbon backbones for posterior use (Krishna et al.1995, zur Nieden et al. 1995), in this case only 'Vermelho 2157' showed a pattern like that. Dell'Aquilla and Spada (1994) reported that there were protein pattern alterations when plants were exposed to high temperatures (40 ºC/48h), especially in relation to normal patterns of imbibition and germination, that is, 20 ºC/24h.

Proline accumulation is associated to different kinds of stress (Marur et al. 1994, Andrade et al. 1995, Pérez-Alfocea et al. 1996) and occurred differentially in the studied material. There were three response patterns to the thermal treatments in the tested material: I) plants that did not increase proline production; II) plants that responded to heat shock only; and III) plants that responded to heat and cold shocks.

Imbibed seeds were metabolically activated but not ready to germinate at high temperatures, e.g. over 30 ºC, and to accumulate proline to protect themselves against the heat stress and prepared to start to grow after the stress was over. However, other kinds of amino groups such as the polyamines were found, although this response seems to be more due to a high temperature stress than to the opposite, although it might be possible to find germplasm with this kind of response.

Recent bred cultivars did not show any response as proline accumulation due to thermal shocks (pattern I - 'IAPAR 57', 'Aporé' and 'IAC-Carioca-Akytã'), whereas 'Vermelho 2157', a recent bred cultivar responsive to both stress temperatures, was placed in pattern III with 'Guarumbé', 'Iratin' and 'Rosinha G2', two wild populations and an old cultivar. 'IAC-Carioca-80SH', 'Rudá', and 'Campeão-1' followed response pattern II (plants responding to heat shock only). In some cases, as in 'Guarumbé' and 'Iratin', proline did not accumulate but decrease when exposed to constant low temperatures (T1, Table 1). Premachandra et al. (1995) reported that an increase in proline concentrations in sorghum lines with resistance to water deficit was lower than in susceptible lines, indicating that proline accumulation could be used as an osmotic protector in susceptible plants against this kind of stress and could also be used as an indicator of salinity injured tissues (Pérez-Alfocea et al. 1996). 'Guarumbé' accumulated proline in response to high temperatures (Tables 1 and 2) and was, according to Souza et al. (2003), the most resistant material tested to drought stress after 10 days without water application - in contradiction to the data presented by Andrade et al. (1995), who tested lines that accumulated proline and were sensitive to drought stress. For instance, Sairam et al. (2002) showed that wheat tolerant lines could be selected by proline accumulation and other biochemical measures.

According to Al-Karaki et al. (1996), there were no differences in proline accumulation among bean species. However, Andrade et al. (1995) showed that in *P. vulgaris* there were differences in this character between different cultivars among the four bean growing types. The data presented in this study showed that increase in proline were different between the cultivated and the landraces and that it could be delayed by supra or sub-optimal temperatures as in Trotel et al. (1996).

Table 1. Proline concentration in bean (*Phaseolus vulgaris* L.) seeds exposed to thermal treatments

Cultivars	Proline accumulation (μg g^{-1}) Thermal treatments				
	— T1 —	— T2 —	— T3 —	— T4 —	— T5 —
Rosinha G2	354 BCa	329 CDEab	255 DEFb	267 EFab	353 Ca
IAC - Carioca 80SH	232 DEb	249 DEFb	395 BCa	376 CDEa	344 Ca
Guarumbé	1005 Ae	1328 Ac	1158 Ad	1499 Ab	3984 Aa
Vermelho 2157	457 Ba	398 BCab	335 BCDb	414 Cab	327 CDb
Iratim	365 BCc	450 Bbc	433 Bc	532 Bab	561 Ba
IAPAR 57	336 CDa	305 CDEa	260 DEFab	297 DEFa	199 Eb
IAC-Carioca-Akytã	349 BCa	322 CDEa	271 DEFab	290 DEFab	205 Eb
Campeão-1	176 Eb	189 Fb	195 EFGb	344 CDEa	197 Eb
Aporé	215 Eb	216 EFb	343 BCDa	304 CDEFab	348 Ca
Rudá	124 Ea	139 Fa	140 Ga	213 Fa	206 Ea

The same letters, lower case in lines and capital in columns, did not differ at P<0.05 by Tukey's test

Table 2. Effect of thermal treatments and proline accumulation in bean (*Phaseolus vulgaris*) seed cultivars

	Proline accumulation (μg g^{-1}) Thermal treatments				
	T1	T2	T3	T4	T5
Time (h)			'IAC - Carioca 80SH' (315 *d*)		
24	272 Aa	280 Aa	280 Ba	280 Ba	147 Ca
48	221 Aa	351 Aa	351 Ba	351 Ba	297 BCa
72	243 Abc	178 Ac	620 Aa	586 Aa	388 ABb
96	192 Ab	187 Ab	331 Bb	289 Bb	546 Aa
			'Rosinha G2' (312 *d*)		
24	320 Aa	260 Ba	260 Aa	260 Aa	187 Ca
48	376 Aab	209 Bb	209 Ab	209 Ab	560 Aa
72	317 Aab	489 Aa	280 Ab	317 Aab	291 BCb
96	405 Aa	356 ABa	272 Aa	283 Aa	376 Ba
			'IAPAR 57'(280 *d*)		
24	336 Aa	262 Aa	262 Aa	262 Aa	150 Aa
48	291 Aa	283 Aa	283 Aa	283 Aa	193 Aa
72	346 Aab	392 Aa	327 Aab	298 Aab	180 Ab
96	372 Aa	285 Aab	167 Abb	346 Aab	274 Aab
			'Aporé'(285 *d*)		
24	226 ABa	173 Aa	173 Ba	173 Ba	223 Ba
48	122 Bb	314 ABa	314 ABa	314 ABa	478 Aa
72	195 ABb	209 Ab	427 Aa	320 ABab	365 ABab
96	317 Aab	167 Ab	458Aa	410 Aa	328 ABab
			'Guarumbé'(1795 *a*)		
24	1991 Aa	1337 Bb	1337 Ab	1337 Bb	1431 Cb
48	907 Bb	1018 Cb	1018 Cb	1018 Cb	1361 Ca
72	560 Ce	1721 Ac	1079 BCd	2428 Aa	1991 Bb
96	560 Cc	1234 Bb	1198 ABb	1210 Bb	11150 Aa
			'Iratim'(468 *b*)		
24	583 Aa	494 ABa	494 Aa	494 BCa	465 Ba
48	291 Bb	318 Bb	318 Ab	318 Cb	508 ABa
72	304 Bb	532 Aa	465 Aab	532 Ba	596 ABa
96	282 Bb	454 ABb	453 Ab	782 Aa	677 Aa
			'Vermelho 2157' (386 *c*)		
24	667 Aa	432 Ab	432 Ab	432 ABb	379 Ab
48	456 Ba	304 Aa	304 Aa	304 Ba	419 Aa
72	303 Ba	462 Aa	339 Aa	428 ABa	374 Aa
96	404 Bab	395 Aab	264 Abc	492 Aa	136 Bc
			'Campeão 1' (220 *e*)		
24	187 Aa	217 Aa	217 Aa	217 Ba	262 Aa
48	196 Aa	156 Aa	156 Aa	156 Ba	221 Aa
72	181 Ab	163 Ab	238 Ab	450 Aa	148 Ab
96	141 Ab	219 Ab	168 Ab	553 Aa	157 Ab
			'Rudá' (165 *f*)		
24	134 Aa	141 Aa	141 Aa	141 ABa	179 Aa
48	128 Aa	113 Aa	113 Aa	113 Ba	284 Aa
72	125 Aa	149 Aa	148 Aa	295 Aa	255 Aa
96	110 Ab	152 Aab	157 Aab	305 Aa	108 Aab
			'IAC-Carioca – Akytã' (288 *d*)		
24	312 Aa	265 Aa	265 Aa	265 Aa	165 Aa
48	312 Aa	289 Aa	289 Aa	289 Aa	183 Aa
72	351 Aa	364 Aa	309 Aa	291 Aa	185 Aa
96	421 Aa	372 Aab	220 Ab	315 Aab	288 Aab

Different letters, capital in the columns and lower case in the lines, and in front of the cultivar names in italic, are different at 5% in Tukey's test

CONCLUSIONS

Proline accumulation is not linear and temperature-dependant. If the response was observed immediately after a long period of exposure, e.g. after 96h exposure to continuous cold or heat, the results could be misinterpreted, leading to false diagnosis of the examined material. To get an accurate idea of what happened, periodical observations of the response are required. In germinating seeds, proline could be used to discover stress temperature responsive lines in beans. The best moment to get a maximum of information is after 24h of exposure to shock temperature.

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Prolina: uso como indicador de estresse térmico em sementes de feijão

RESUMO - *Estresse devido à temperatura pode levar a diversas desordens metabólicas durante todo o ciclo de vida das plantas, incluindo-se aí a germinação. Para verificar o efeito de altas ou baixas temperaturas, constantes ou em ciclos alternados, 10 genótipos de feijoeiro (8 cultivares e duas variedades crioulas) foram germinadas em ensaios para quantificação de prolina. Tais ensaios foram conduzidos em câmaras climáticas sob cinco tratamentos térmicos: T1 - temperatura sub-ótima (96h a 8 ºC); T2 - choque frio (48h a 18 ºC seguidos de 48h a 8 ºC); T3 - temperatura ótima (96h a 18 ºC); T4 - choque quente (48h a 18 ºC seguidos de 48h a 37 ºC) e; T5 - temperatura supra-ótima (96h a 37 ºC). Prolina foi quantificada por espectrofotometria a 520nm. Houve respostas a diferentes ambientes para cada genótipo. Prolina pode ser usada, em sementes em germinação, para identificar linhagens capazes de responderem a temperaturas estressantes, como ocorreu com 'Guarumbé'.*

Palavras-chave: *Phaseolus vulgaris*, choque de calor, choque frio, germinação, aminoácidos.

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