ON THE INFLUENCE OF THE TEMPERATURE AND PH OF THE INCUBATION MEDIUM ON THE ACTIVITY OF TOTAL AMYLASE IN SOME SPONTANEOUS AND CULTIVATED *POACEAE*

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Key words: total amylase, optimum pH, optimum temperature, graminaceae
Abstract: The paper analyzes the influence of the temperature and pH of the incubation medium on the activity of total amylase, in four species of spontaneous and cultivated graminaceae, namely: *Panicum miliaceum*, *Setaria pumila*, *Festuca pratensis* and *Sorghum sudanense*. The results obtained evidence, on one side, the decisive influence of temperature and pH of the incubation medium on the rate of the enzymatic reactions and, on the other, the existence of certain differences, from one species to another, as to the optimum action values of temperature and pH.

INTRODUCTION

As generally known, the process of germination assumes enzymatic degradation of the reserve substances from seeds. Consequently, the reserve proteins are hydrolyzed by proteases and then decomposed in more and more simple substances, such as: peptides, amino acids, amides, ammonium, while the lipids get transformed - under the action of lipase - in glycerin and fatty acids; in a subsequent stage, glycerin is transformed into sugars, while the fatty acids enter the cycle of tricarboxylic acids, in embryo's breathing process (BURZO *et al*., 1999).

Another well-known fact is that, in conditions of biological repose, the enzymatic activity is almost wholly reduced and that, with the absorption of the environmental water during germination, the entire enzymatic equipment is activated, for assuring the energy required by the various metabolic processes (CIORNEA and VASILE, 2007).

MATERIALS AND METHOD

The experiments have been developed on germinated caryopses of *Panicum miliaceum*, *Setaria pumila*, *Festuca pratensis* and *Sorghum sudanense.*

Determination of the enzymatic activity was made by the Noelting - Brenfeld method, based on the reduction of the free maltose resulting from the enzymatic hydrolysis of starch, with 3,5 - dinitrosalicylic acid, with formation of 3-amino-5-nitrosalicylic acid, orange in color, determined colorimetrically at 540 nm; in a final stage, the results were statistically processed (ARTENIE and TĂNASE, 1981; FOWLER *et al*., 2000).

RESULTS AND DISCUSSION

The enzymatic activity is decisively influenced by the temperature and pH of the incubation medium; consequently, for attaining an as high reproducibility degree of the experimental results as possible and for avoiding any errors, induced by possible differences in the operation mode, a first series of experiments was devoted to the determination of the optimum conditions of pH and temperature of the amylolytic activity in the graminaceae species under investigation.

Literature data (COJOCARU *et al*., 2007) evidence the apical part played by the pH action of the enzymatic catalysis, the biological catalysts being especially sensible to the large variations of this parameter.

Temperature may have a considerable influence on the enzymatically catalyzed reactions, by modifying the enzyme stability and its affinity towards the substrate, the scission rate of the enzyme-substrate complex, as well as the affinity towards effectors. Most of the enzymes are biomolecules with low thermal stability, higher catalytic activities being recorded over relatively narrow temperature domains, more frequently with enzymes of animal origin. In the case of vegetal enzymes, the thermal intervals over which they develop their maximum catalytic ability are somewhat larger, once, in plants, the thermal homeostasis occurring in animal organisms is not so strict.

The effect of temperature over the stability of the enzymes is determined through pre-incubation of the enzyme, separated from its substrate, at the temperature at which the

determination is performed. The literature of the field evidences the special part played by the calcium ion in assuring the thermostability of amylases (KADZIOLA *et al*., 1998; XAVIER *et al*., 2003). Thus, substitution of the calcium ions from the enzymatically-active center induces modifications at the level of the secondary and tertiary structure (BUSH *et al*., 1989), alongwith lower thermostability (NIELSEN *et al.*, 2003) and enzymatic activity (MACGREGOR *et al*., 2001).

The experimental data obtained, evidencing the influence of temperature on the activity of total amylase in millet, bristle grass, hair grass and Sudan grass, and plotted graphically in Figure 1, show that the total amylasic activity oscillates within quite large limits at various temperature values, the enzyme attaining its maximum activity over a temperature interval between 35 and 45°C. Such results agree with those described in literature, which attest that, in the case of rice, the optimum action temperature ranges between 45 - 50°C while, in lentil cotyledons, it is of 40°C (SHAHA *et al.*, 2004) and, in germinated **millet** seeds (***Panicum miliaceum***) the optimum temperature would be around **45°C** (GIMBI *et al*., 2002), while the optimum temperature value of α-amylase action registered in the seed vessel of the *Borassus indica* species being of 37°C (RAO *et al*., 2005).

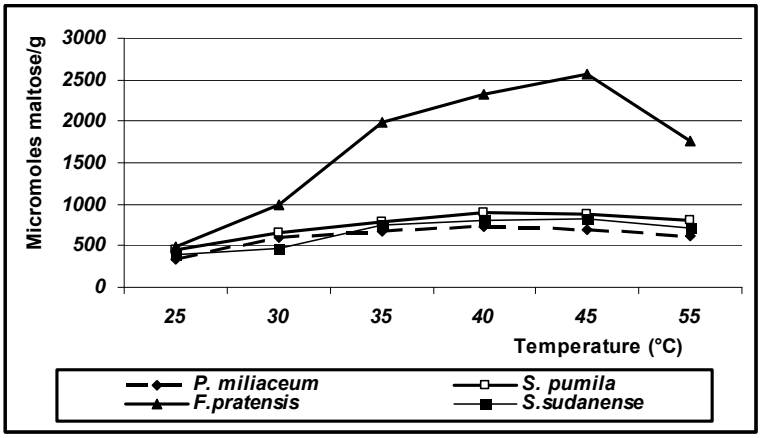

Fig.1. Influence of the incubation medium temperature on the activity of total amylase

Generally, the pH may affect the enzymatic activity either irreversibly - when denaturation of the protein-enzyme macromolecules occur - or reversibly, by influencing the ionization degree of the substrate, of the enzyme or of the enzyme-substrate complex. For all enzymes known up to now, the graphical representation of the dependence of the enzymatic reaction speed on the pH of the incubation medium appears as a Gauss curve, the maximum catalytic activity being registered at a well-determined pH value, known as optimum action pH, which differs from one enzyme to another (COJOCARU, 1997; COJOCARU *et al*., 2007).

The influence of pH on the stability of the enzyme is tested by exposure to different values of the hydrogen ions concentrations, by readjusting the pH to the optimum action value, and by testing the catalytic activity.

The present investigations analyze the manner in which the ionization state of the enzyme or of the enzyme-substrate complex influences the rate of the enzymatic reaction. To this end, the samples were incubated at 40°C, for 30 minutes, in aqueous media with various pH values, obtained by addition of either acetic acid or sodium hydroxide to the acetate buffer

solution, on maintaining constant all the other reaction conditions; the obtained data are plotted in Figure 2.

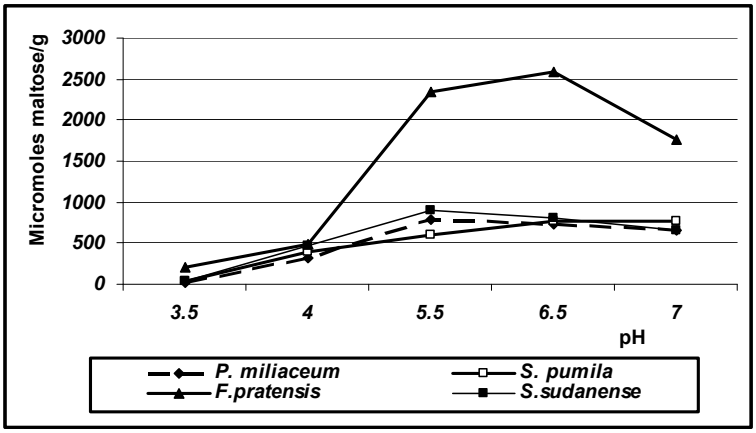

Fig.2. Influence of the incubation medium pH on the activity of total amylase

Data referring to the dependence of the enzymatic activity on the pH of the incubation medium show that the activity of total amylase in germinated caryopses of *Panicum miliaceum*, *Setaria pumila*, *Festuca pratensis* and *Sorghum sudanense* is maximum over a pH interval between 5.5 - 6.5, which agrees with literature information, according to which, in the case of **millet**, the optimum action pH of α-amylase is of **5.4**, while β-amylase has an optimum pH of **6.0** (GIMBI and KITABATAKE, 2002) and the optimum action pH of the α-amylase from coffee beans is between 4.5 - 5.2 (VALENCIA *et al*., 2000).

A first objective considered in the determination of amylase activity in the species under study was plotting of the standard curve for converting the extinction units (Fig. 3). To this end, a series of reference samples - in which the concentration in maltose varied between 0.2 - 1.8 mg - has been employed. The values of extinction have been read at a wavelength equal to 540 nm.

On the basis of the graph, the regression straight line has been drawn and its regression equation has been calculated. According to the equation, the amounts of maltose corresponding to the samples subjected to analysis have been subsequently established.

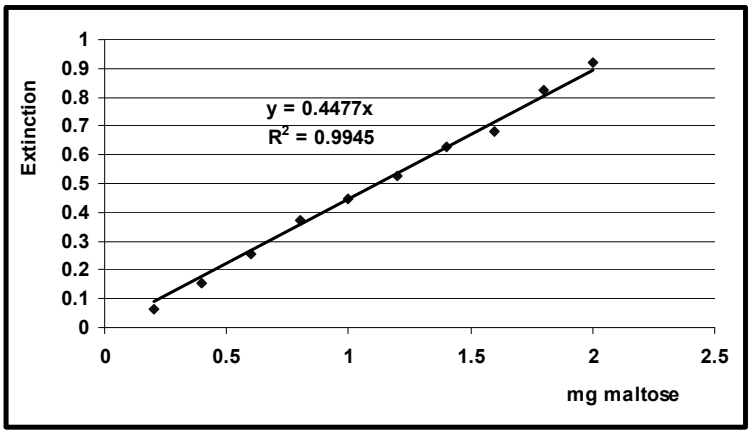

Fig.3. Standard curve for dosing of maltose (original)

Consequently, for all species under study, the amylase activity was determined on samples taken over at intervals of 24 hours, along ten germination days. During germination of the millet seeds, the activity of total amylases suffers certain modifications, comparable to those described in literature (LAURIÈRE *et al*., 1992), more precisely, the enzymatic activity is influenced by the germination time.

As to the activity of total amylase in germinated *Panicum miliaceum* caryopses, three parallel determinations were made for each of the samples under analysis, followed by statistical processing of the results, calculation of the standard error, standard deviation, variation coefficient of the average value, precision coefficient of the mean value as well as the superior and inferior limits of the confidence intervals.

In millet caryopses occurring in biological repose, moment zero, the activity of total amylase attains the minimum threshold, with values ranging between 58.873 and 60.561 μM maltose/g, and a mean value of 60.185 μM maltose/g. After 24 hours of germination, the total amylase records an average activity of 115.307 μM maltose/g, which progressively increases, up to the maximum value (812.707 μM maltose/g) recorded in the 5th germination day.

Starting with the 7th germination day, the enzymatic activity progressively decreases, up to 508.509 μM maltose/g at 80.876 μM maltose/g, respectively (the minimum value of total amylase from the millet caryopses under analysis) (CIORNEA *et al*., 2006 a) (Fig. 4).

The above discussed data show that, in the first five germination days, total amylase records an accelerated increase in its catalytic activity, up to an almost double value - *versus* the reference - after 24 hours, and up to almost 14 times higher after 120 hours of germination, after which it progressively decreases until the 10th day, when it attains a value with the same order of magnitude as the reference. Such dynamics of the total amylasic activity supports the assumption that mobilization of reserve starch, for assuring the energy required by the metabolic processes, starts in the very first hours of the germination process, even if, in the beginning, the catalytic activity is modest.

The gradual decrease of the amylolytic activity in the second stage of the interval taken into study might be explained by a gradual reduction of the starch amount and, probably, by the initiation of the photosynthetic process, known as assuring itself the precursors for the metabolic processes.

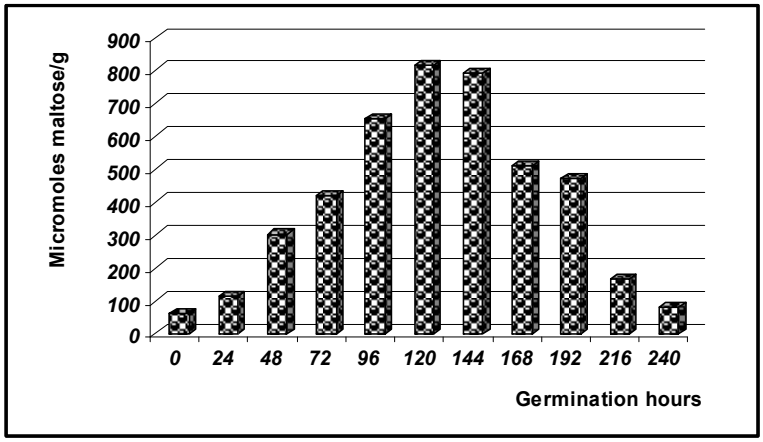

Fig.4. Total amylase activity (μM maltose/g) in *Panicum miliaceum* germinated caryopses

By means of the average values and of the standard deviation, there have been subsequently calculated the superior and inferior confidence limits, on the basis of the critical value *t* for $\alpha = 0.05$ and n-1 degrees of freedom, data synthesized in Figure 5.

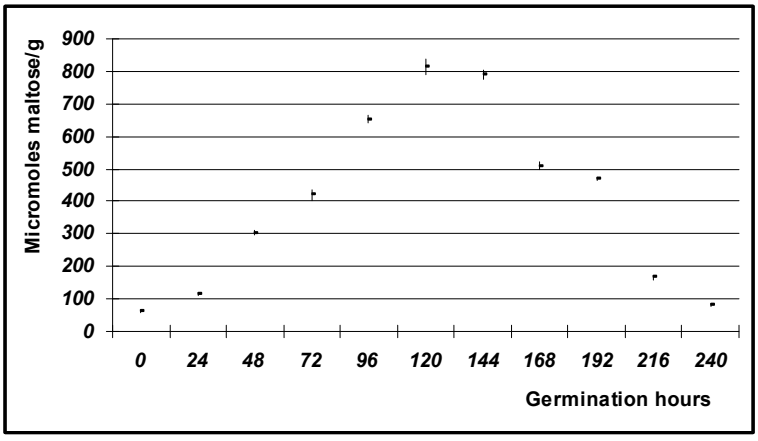

Fig.5. Confidence intervals of total amylase in *Panicum miliaceum*

Figure 5 evidences that the confidence limits of the activity of total amylase are extremely narrow for all germination hours taken into study. The largest variability intervals are recorded at 120 germination hours, a case in which the limits range between 788.497 - 836.917 μM maltose/g.

Unlike *Panicum miliaceum*, in which the maximum total amylasic activity is registered four days after the beginning of the germination process, and maintained at extremely high values over the 48 - 144 hour interval, in the case of *Setaria pumila*, the maximum value of the total amylasic activity occurs in the 6th day, when the recorded value is double than the one of the 5th and, respectively, 7th day.

Starting with the first 24 hours of germination, the activity of total amylase increases progressively from one day to another. Thus, the enzymatic activity attains values of 105.236 μM maltose/g in the first germination day, up to a maximum value of 786.228 μM maltose/g in the 6th germination day, after which it gradually decreases (421.977 μM maltose/g at 192 germination hours, up to 83.458 μM maltose/g at 240 germination hours) (Fig. 6).

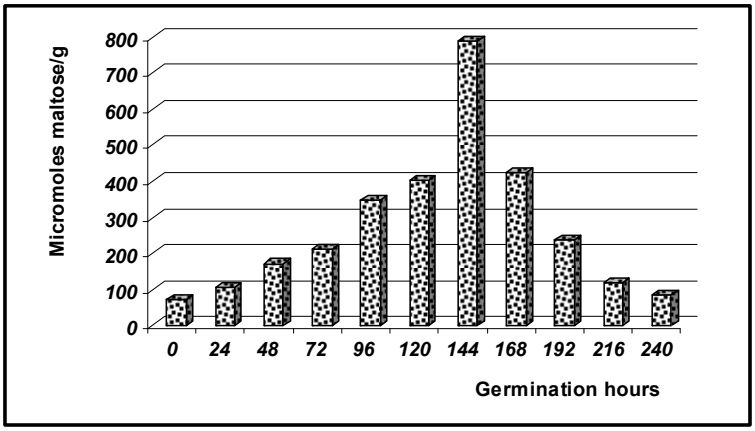

Fig.6. Total amylase activity (μM maltose/g) in *Setaria pumila* germinated caryopses

In bristle grass, the interval with the highest confidence appears at 144 hours of germination, along the other germination periods taken into study the limits of the intervals being extremely narrow (Fig. 7).

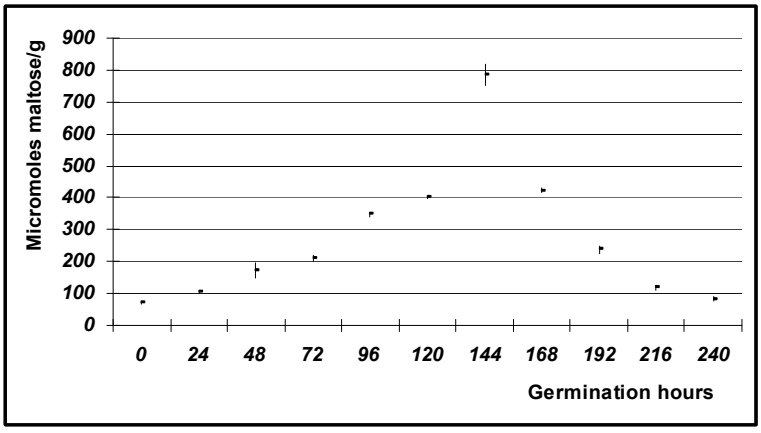

Fig.7. Confidence intervals of total amylase in *Setaria pumila*

Another objective of the present study referred to the determination of total amylasic activity in *Festuca pratensis*, a species in which - according to the results obtained - the activity of total amylase, analyzed on samples taken over at intervals of 24 hours, along ten germination days, suffers certain modifications.

Analysis of the experimental results on the dynamics of total amylase in *Festuca pratensis* shows a much higher enzymatic activity in this species, comparatively with the previously investigated one, characterized by a progressive increase up to the 6th germination day.

Consequently, if in the first germination day, *i.e.*, in the beginning of germination (moment zero), the enzymatic activity in the impregnated sample was minimum, occurring - on the average - around a value of 186.7 µM maltose/g, after the establishment of the optimum conditions - through absorption of the environmental water and membrane permeabilization -, the enzymes involved in the catabolysis of the reserve substances are reactivated for producing the precursors necessary in biosynthetic processes (CIORNEA *et al*., 2006 b). In the 6th day, the activity of total amylase attains the maximum threshold (2451.9 µM maltose/g), after which it slightly decreases up to 240 germination hours (563.539 µM maltose/g) (Fig. 8).

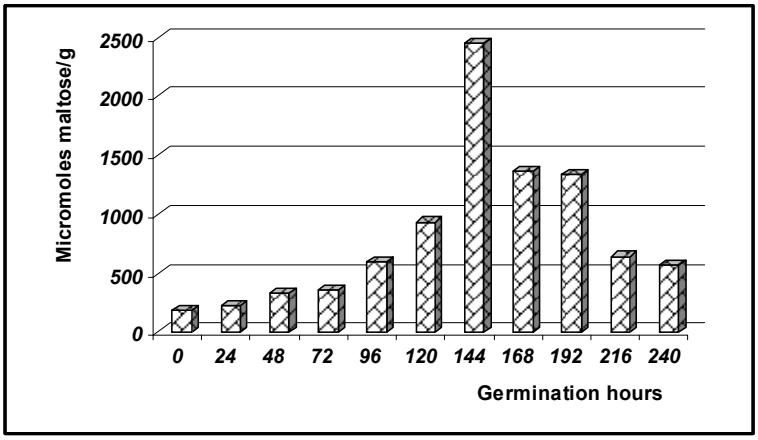

Fig.8. Total amylase activity (µM maltose/g) in *Festuca pratensis* germinated caryopses

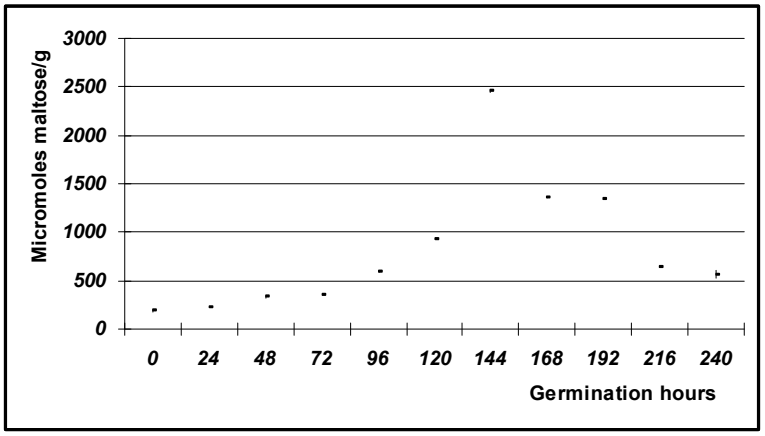

Fig.9. Confidence intervals of total amylase in *Festuca pratensis*

In Sudan grass caryopses occurring in biological repose (moment zero), the activity of total amylase reaches its minimum threshold, taking values between 65.189 - 66.123 μM maltose/g, with an average value of 65.557 μM maltose/g.

After 24 hours of germination, total amylase records an average activity of 111.491 μM maltose/g, which progressively increases up to a maximum value, attained in the 7th germination day (1407.702 μM maltose/g), to be followed by a progressive decrease up to 410.947 μM maltose/g (at 240 germination hours) (Fig. 10).

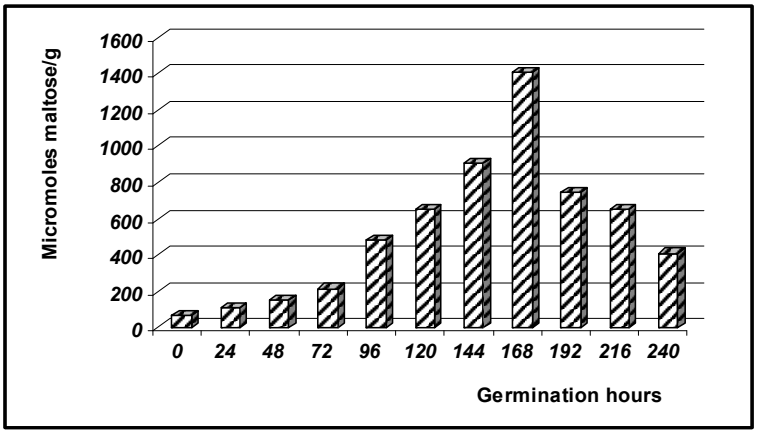

Fig.10. Total amylase activity (μM maltose/g) in *Sorghum sudanense* germinated caryopses

As graphically plotted, for all samples under investigation, the confidence intervals are extremely narrow, with the only exception of the 7th day, when the interval is somewhat larger (846.306 - 876.669 μM maltose/g) (Fig. 11).

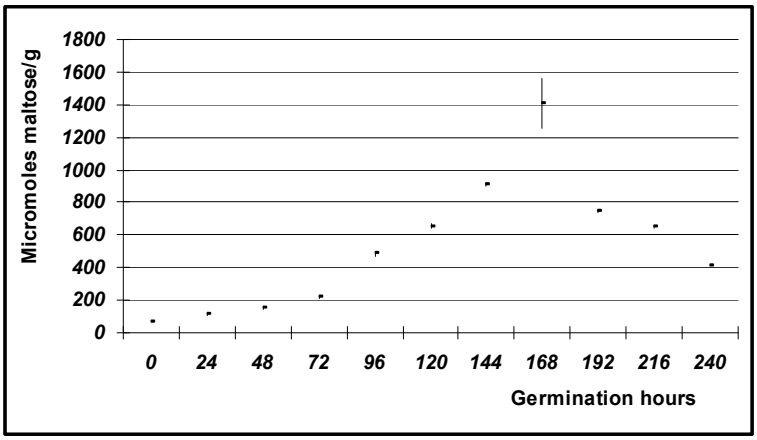

Fig.11. Confidence intervals of total amylase in *Sorghum sudanense*

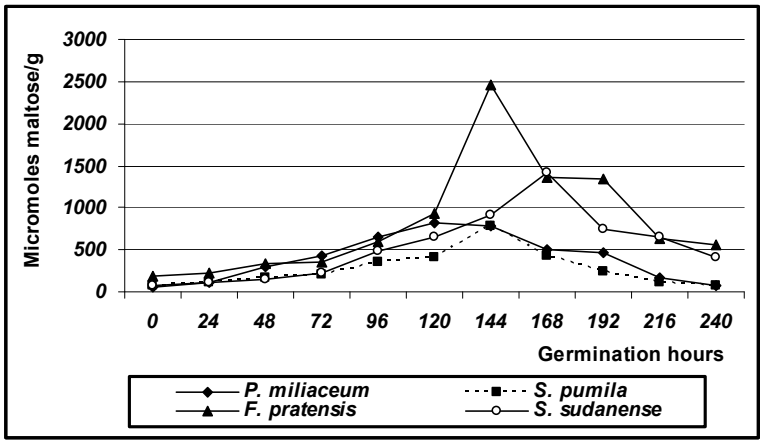

Fig.12. Graphical representation of total amylase activity in some cultivated and spontaneous graminaceae

CONCLUSIONS

For all species taken into study, the optimum pH of the total amylolytic activity occurred in the weakly acid area (pH values between 5.5 and 6.5), although the total amylasic activity remains at high values over a somewhat larger pH interval (5.5 - 7.0), which agrees with the literature data according to which the optimum pH of the amylases is significantly different in the vegetal world, oscillating between 4.5 and 8.0.

An even larger range of values, oscillating between 30 and 55°C, has been recorded for the optimum temperature of the total amylase action, which suggests the possible utilization of vegetal amylases in various biotechnological processes.

Along the first 10 days of the germination process, the total amylasic activity shows a Gauss-type dynamics in all species taken into study, certain differences being registered as to the moment in which the maximum activity is attained. Thus, in *Panicum miliaceum*, the maximum activity of total amylases was registered at 120 hours of the germination process, in *Setaria pumila* and *Festuca pratensis* - at 144 hours, and in *Sorghum sudanense* - at 168 germination hours.

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