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Full Length Research Paper

Analysis of protein by colorimetric and computer color based intensity measurement method with anatomical sections of gram (*Cicer arietinum*) stem at three stages of growth

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This research work is based on the establishment of new and easy techniques for the analysis of total proteins from anatomical sections. The proteins were measured from three different growth stages of stems of gram (*Cicer arietinum*). Firstly, protein was measured by colorimetric method and then analyzed from the cross sections of the same part of plant, sections were then stained with two different dyes and after that color intensity of stained sections was measured through computer software. Results of Lowry's and Bradford methods showed that the third stage of both plants have a maximum amount of proteins. The staining period of dye was also established, the staining of sections (stems of gram) with all dyes gave maximum amount of protein at 2 h staining when compared to 1, 3 and 24 h of staining periods. The results of the computer analysis showed more concentration of protein content when compared to colorimetric methods in the visible regions. By this method, we can see the migration, destination and distribution of different compounds from the root to the top of the plant in the near future.

Key words: Colorimetric methods, stained anatomical sections, computer software.

INTRODUCTION

Gram (*Cicer arietinum*) belongs to the Fabaceae family; its seeds, stems, rachis and leaves are used for food containing a good amount of protein. Protein is a nitrogenous compound (Stayanarayana and Chakrapati, 2007; Jain et al., 2006) that is essential for all living organisms. It is synthesized in its leaves and green stems and then mobilized to the seeds and fruits (Schiltz et al., 2005). During this movement, different types of proteins are synthesized in leaves, which may be absent in seeds or fruits (Schiltz et al., 2005; Salon et al., 2001; People and Dalling, 1988). In other research works, the focus has been on the synthesis of different proteins in leaves during the growth period, but in this research, only water soluble proteins were analyzed by using colorimetric methods and computer color intensity based method, through anatomical sections of gram at three different growth stages.

Microscopic and anatomical techniques of analysis are old and beneficial (Haseloff, 2003; Esau, 2002; Pandey, 2002; Chandurkar, 1989) but in modern medical science, different disorders are analyzed in anatomy with the help of computer software (De Matos et al., 2006; Arrasate and Finkbeiner, 2005; Zapletal et al., 2004; Buyya et al., 2003; Tonar et al., 2003; More and Jones, 2002) for the observation and analysis of any compound through anatomical sections, specific dyes are needed. In this work, two protein dyes were used, in other words, Coomassie Brilliant Blue G-250 and Lawsone dye were used. Coomassie brilliant blue is what is commonly used

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in Bradford's method and electrophoretic techniques for the staining of protein (Sawhney and Randhir, 2006; Krohn, 2002; Tal et al., 1985; Moller et al., 1993). In this work, Coomassie Brilliant Blue (CBB) dye was used for the staining of double protein in anatomical sections of stem analysis. Lawsone dye is a natural dye which is obtained from Henna (*Lawsonia inermis*), which is commonly used for the dyeing of hair, nails and skin protein (Petkewich, 2006; Wallis, 2005; William, 2002; Dweck, 2002; Nikkel, 2001). It is also recorded that lawsone dye was also used in Electrophoresis for the staining of protein by Ali and Sayeed (1988) but in this work first pure lawsone dye (Ls) was used for the staining of protein and then (hple1) was used for the staining of protein and color intensity of section was measured by computer software analysis.

MATERIALS AND METHODS

Protein analysis by colorimetric methods

Collection of gram samples

Gram (*C. arietinum*) was sown in the first week of December, 2008 by drilling method in the experimental field of the Institute of Plant Sciences, University of Sindh, Jamshoro, Sindh, Pakistan. Plot size for the crop was 10 × 10 m and normal agronomic practices were observed from sowing to harvest. Stem samples were collected at three different periods, which is, 2 months of (pre-flowering stage), 3 months of (flowering stage) and 4 months of (fruiting stage) denoting G-1, G-2 and G-3. At each stages of growth, stem samples were collected from the lower, middle and upper part of the same plant and replicated four times.

Extraction of protein from samples

For this process, 1 g of each sample (G-1, G-2 and G-3) was grinded in pestle mortar with 2.0 ml of 0.1 M phosphate buffer at pH 7.5. These samples were kept overnight for complete extraction of protein, then the volume was made up to 5.0 ml for each sample. These were then centrifuged at 16.163 g for 20 min and repeated for the second time. The supernatant was used for protein analysis and residue was discarded (Sawney and Randhir, 2006).

Procedure for Lowry's method

1.0 ml of supernatant from each of the aforementioned samples was added to 5.0 ml of alkaline copper sulfate reagent, mixed thoroughly and kept for 10 min. and 0.5 ml of Folin's reagent was later added In order or it to develop color. It was again left for another 30 min and the concentration read with the recording absorbance at 660 nm against blank. The blank was prepared by taking 1 ml of 0.5 NaOH in place of the sample in the cuvette. Bovine serum albumin was used to draw a standard curve and the amounts of protein in different samples were estimated (Sawhney and Randhir, 2006; Lowry et al., 1951).

Procedure for Bradford's method

1.0 ml of the supernatant was mixed with 1.0 ml of phosphate buffer and 5 ml of Bradford's reagent was added and absorbance read at595 nm as against the blank reagent. A standard calibration curve was drawn by using bovine serum albumin as a standard. From the standard curve, the concentration of protein in the samples was obtained (Sawhney and Randhir, 2006; Bradford, 1976).

Protein analysis by computer software methods

The following parameters are required for the measurement of color intensities of 2D images (cross sections of plant) and they include:

1. Binocular microscope.
2. Computer (P-iv)
3. USB camera (Pixel 480 × 640)
4. Resolution of monitor. 1024 by 768 pixels
5. C.S of gram (*C. arietinum*).
6. Specific dyes for protein, that is, Coomassie and Lawsone dye.
7. Color intensity measurement computer software.

Computer software

This software was invented by the first author during his Doctoral thesis research work for the measurement of color intensities of unstained and stained plant's sections (2D image). With the help of analytical balance, different sections were weighed and their known intensities fixed in the program of the software, thus, showing the weight of the section. Minimum weight acceptable is 0.1 mg while the maximum is 0.5 mg. In this work, all sections were observed at 0.1 mg weight. After cutting series of sections, only 0.1 mg of sections was selected for all the observations. First of all, average Background of Optical Density (BOD): which is Intensity of screen of window without plant section was measured and then calculated with the Optical Density (OD) (section) of which white is the color, that corresponds to the maximum Optical Density (554.25 ou/μm^2) that is sum of Red, Green and Blue color intensities. (De Matos et al., 2006; Heydorn et al., 2000). The results showed that, 554.25 ou/μm^2 is the constant value which is the sum of the optical density of red, green and blue; where the color Black is absent. Therefore, the optical density values considered by the Computer software formula decrease in the scale in which the high values correspond to the colors that are visually clear. The total intensity (TI) of color was measured using the formula:

$$IT_{2Dimge} = 554.25 \frac{(554.25 \times \sum OD)/ \sum BOD)\ ou/\mu m^2}{\sum BOD}$$

(De Matos et al., 2006; Heydorn et al., 2000).

After calculating the total intensity (TI) of each section, the TI of Stained image was subtracted from the TI of the unstained image named as "Total intensity of Dye" (TID) only. That is, TID = TI of unstained image – TI of stained image. The value of TID showed the amount of protein when Coomassie and Lawsone dye where for the analysis of protein. So TID divided by TI of weighted image (0.1 mg), which is fixed in Software, obtained the % of protein concentration:

$$\text{\% of Protein} = \frac{TID}{\text{TI of weighted image}} \times 100$$

Preparation of sections for microscope

A Cross section of Gram (G-1, G-2 and G-3) was cut down with blade-trig (Haseloff, 2003) before the staining of each plant sections, and then placed on the stage of a Microscope (XSN 107,

China). The microscope was then connected to the computer through digital a Camera (ETUSB-2710) by using a USB port. 25 × magnifications were used for all observations. The Cross sections of plants which appeared on the screen of the computer monitor were saved as unstained 2D images data in computer memory then all plant sections were stained in the following dyes.

Dyes for plant sections staining

The following dyes were prepared for the staining of anatomical sections of gram:

1. Coomassie Brilliant Blue G-250 (CBB dye) (Willaim, 2002).
2. Lawsone dye
a) Pure Lawsone dye (Ls dye).
b) Lawsone dye: Extracted from Henna powdered leaves for one hour (Hple1)

Staining of section by coomassie brilliant blue dye (CBB Dye)

Preparation of CBB dye

The Coomassie Brilliant Blue (G-250) was dissolved in 12.5ml of pure ethanol add 25 ml of 85% phosphoric acid, after that the volume was made up to 250 ml with distilled water. Distaining solution was prepared with Methanol and 7% acetic acid (1: 1) (Santiago, 1989). Two Petri dishes were taken, labeled as A and B. A petri dish contained staining CBB dye and B Petri dish contained distain solution.

Staining of C.S of gram (G-1, G-2 and G-3) with CBB dye

Cross sections of Gram stem (G-1, G-2 and G-3) were stained in the CBB dye separately for 1, 2, 3 and 24 h respectively, at room temperature and observed under microscope and 2D images. Data from the observation was saved in two forms, unstained and stained in a computer memory and analyzed by computer software. (Pons et al., 1997; Sawhney and Randhir, 2006; Heydorn et al., 2000).

Staining of sections with pure Lawsone dye (Ls Dye)

Preparation of Ls dye

For this observation, 2 g of Lawsone dye was mixed with distilled water and the volume adjusted to 100 ml, which gave 2% pure Lawsone dye (Ali and Sayeed, 1988). Then, two petri dishes where labeled A and B with Petri dish A containing 2% of Lawsone dye and Petri dish B containing distilled water which was used for distaining.

Staining of C.S of gram (G-1, G-2 and G-3) with Ls dye

A Cross section of Gram stem (G-1, G-2 and G-3) were stained in 2% Ls dye (pH-4) for 1, 2, 3 and 24 h respectively, separately at room temperature and then observed under microscope and then 2D image data were saved in two forms unstained and stained data in computer memory. Protein concentration was analyzed by computer software (Pons et al., 1997; Ali and Sayeed, 1988; Catherine, 2005).

Staining of C.S of gram (G-1, G-2 and G-3) with hple1

A Cross sections of Gram stem (G-1, G-2 and G-3) were stained in 2% Hple1 (pH-4) for 1, 2, 3 and 24 h respectively, separately at room temperature and observed under microscope and 2Dimage data were saved in unstained and stained data form in computer memory and protein was analyzed by computer software (Heydorn et al., 2000).

RESULTS

Protein determination at three stages of growth of gram (G-1, G-2 and G-3) by colorimetric method

The protein of stem was determined at three different stages of growth by Lowry's and Bradford quantitative methods. G-1 gave 0.55 mg/100 g using Lowry's method and 0.46 mg/100 g was realized from Bradford's method. In the second analysis, 4.66 mg/100 g protein was determined from G-2 using Lowry's method and 4.76 mg/100 g from Bradford' method while G-3 62.50 mg/100 g were realized from Lowry's method and 62.55 mg/100 g from Bradford's method (Figures 1 and 2).

Protein analysis from three stage of growth of gram (G-1, G-2 and G-3) by computer software

First stage of growth of gram (G-1)

The sections of G-1 were stained in three different types of dyes, that is, CBB dye, Ls dye and Hple1 for some time intervals, ranging from 1, 2, 3 and 24 h respectively, at room temperature. The best recovery of protein contents in G-1 appeared after 2hrs of staining in either CBB dye (2.11 mg/100 g), Ls dye (1.20 mg/100 g) or in Hple1 (1.01 mg/100 g) while sample stained for 1, 3 and 24 h respectively, in different dyes revealed lower concentration of protein content

The results from the analysis is as follows; CBB dye produced 1.2, 2.04 and 1.04 mg/100 g, in Ls dye, it was 0.26, 0.42 and 0.22 mg/100 g and in Hple1 0.97, 0.94 and 0.7 mg/100 g respectively (Figure 3). Furthermore, the results showed (Mean ± SEM) that the concentration of protein contents was remarkably high in G-1 stained with CBB dye (1.60 ± 0.27 mg/100 g) compared to those stained with Ls dye (0.52 ± 0.22) and Hple1 (0.90 ± 0.067). Further observation using ANOVA showed that there were no significant differences ($p > 0.05$) in protein contents in three staining dyes. However, when Least Significantly Difference (LSD at rejection level 0.05) in the mean of protein content revealed significant differences ($P<0.01$) in the concentration of protein content with CBB dye stained samples compared to that of Ls dye and Hple1 whilst Ls dye and Hple1 stained sample revealed relatively similar samples ($P > 0.05$) concentration of protein contents (SAS Institute Software).

The second stem growth of stages gram (G-2)

During the analysis, sections of G-2 stained in three dyes

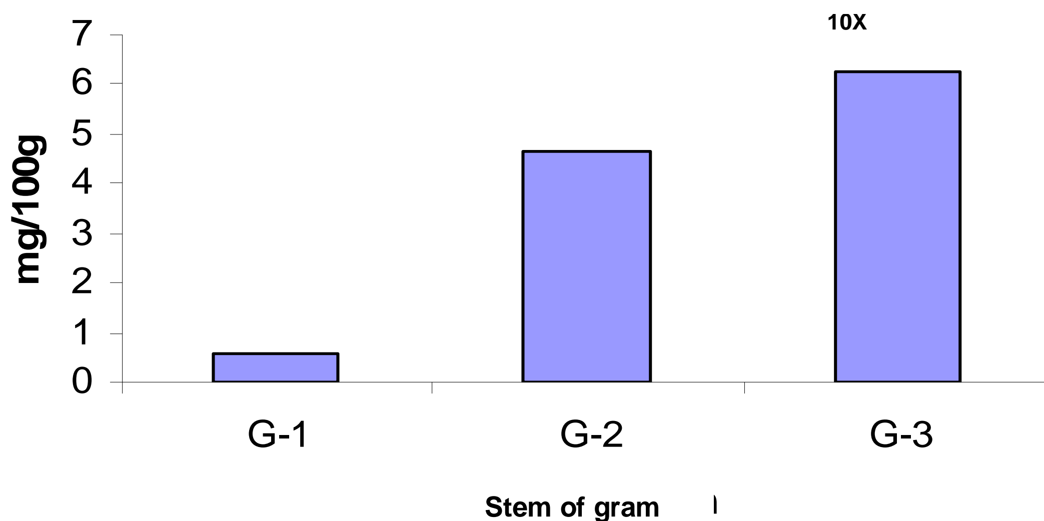

Figure 1. Stem of gram: Protein analysis by Lowry's method from three stages of growth, result of G-3 multiply by 10.

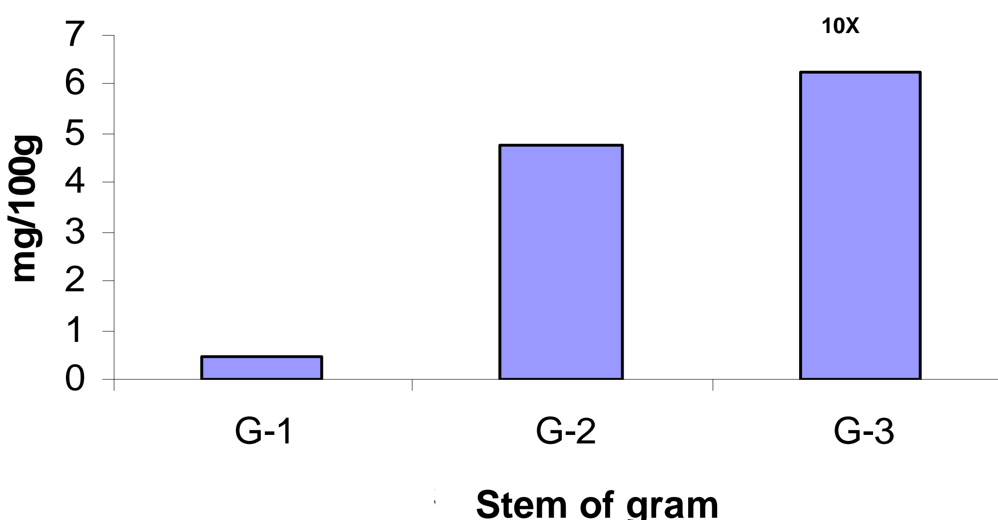

Figure 2. Stem of gram: Protein analysis by Bradford method from three stages of growth, result of G-3 multiply by 10.

that is, CBB dye, Ls dye and Hple1 for different time intervals, that is, after 1, 2, 3 and 24 h respectively, at room temperature for observation. The color intensity of three dyes was analyzed by computer software for the estimation of protein content. It was observed that the staining time in different dyes (CBB dye, Ls dye and Hple1) gave remarkable variation in total protein content of the samples. The best staining was observed after 2 h either in CBB dye (6.11 mg/100 g) Ls dye (5.0 mg/100 g) and Hple1 (4.88 mg/100 g) where staining after 1, 3 and 24 h respectively, with different dyes revealed lower concentration of protein content that is, CBB dye 3.81, 5.87 and 3.03 mg/100 g Ls dye 4.11, 4.66 and 4.15 mg/100 g and Hple1 4.10, 4.22 and 4.05 mg/100 g) respectively (Figure 4). Further more, the result showed (Mean ± SEM) that the concentration of protein contents was not high in G-2 sample staining with three dyes for example CBB dye (4.70 ± 0.76 mg/100 g), Ls dye (4.48 ± 0.21) and (4.31 ± 0.19 mg/100 g). Statistical values by ANOVA showed that there was no significant difference ($P > 0.05$) between the three types of dyes used. However, Least significant difference (LSD at rejection level of 0.05) in the mean of protein content showed that there was no significant ($P > 0.05$) difference among the means of three staining dyes of protein in G-2 structure (SAS Institute Software).

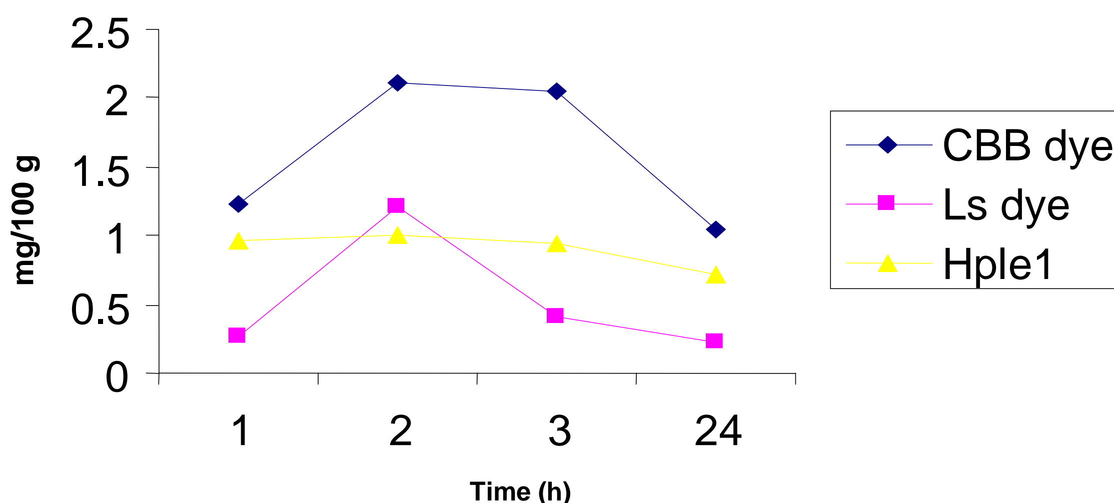

Figure 3. Comparison of staining of C.S of gram (G-1) with CBB dye Ls dye and hple1 for the assessment of protein for different time intervals at room temperature.

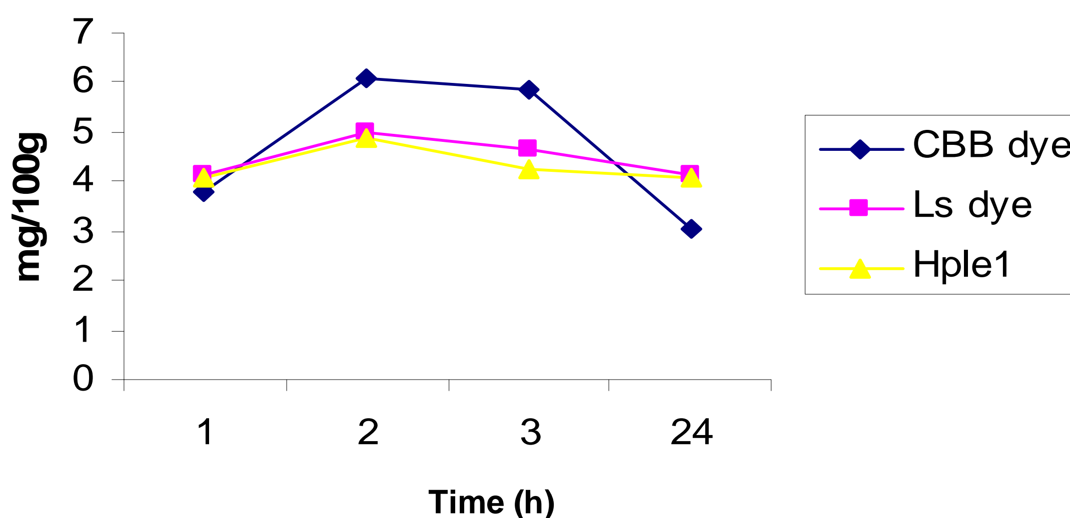

Figure 4. Comparison of staining of C.S of gram (G-2) with CBB dye Ls dye and hple1 for the assessment of protein for different time intervals at room temperature.

The third stem growth of stage gram (G-3)

The analysis was carried out with sections of G-3 stained in three dyes that is, CBB dye, Ls dye and Hple1 for different time intervals that is, 1, 2, 3 and 24 h respectively, at room temperature. Computer software was then used for the determination of total protein in G-3. It was observed that the staining time in different dyes (CBB dye, Ls dye and Hple1) did not have remarkable variation in G-3 samples. The maximum recovery of proteins content in G-3 was observed at 2 h either with CBB dye (70.33 mg/100 g), Ls dye (64.2 mg/100 g) and Hple1 (63.89 mg/100 g while sample stained for 1, 3 and 24 h respectively, in different types of dye, revealed lower concentration of protein content that is, CBB dye 50.55, 69.78 and 60.56 mg/100 g, in Ls dye 61.83, 63.70 and 61.53 mg/100 g and in Hple1 60.69, 62.58 and 60.18 mg/100 g respectively (Figure 5). Further more, the result showed (Mean ± SEM) that the concentration of protein was less in G-3 sample stained with Hple1 (61.83 ± 0.86) compared to those stained with CBB dye (62.80 ± 4.66) and Ls dye (62.81 ± 0.66 mg/100 g). Statistical values by ANOVA showed that there is no significant difference ($P > 0.05$) between three dyes. However, least significant difference (LSD at 0.05 rejection level) also showed that there was no significant ($P > 0.05$) difference among the

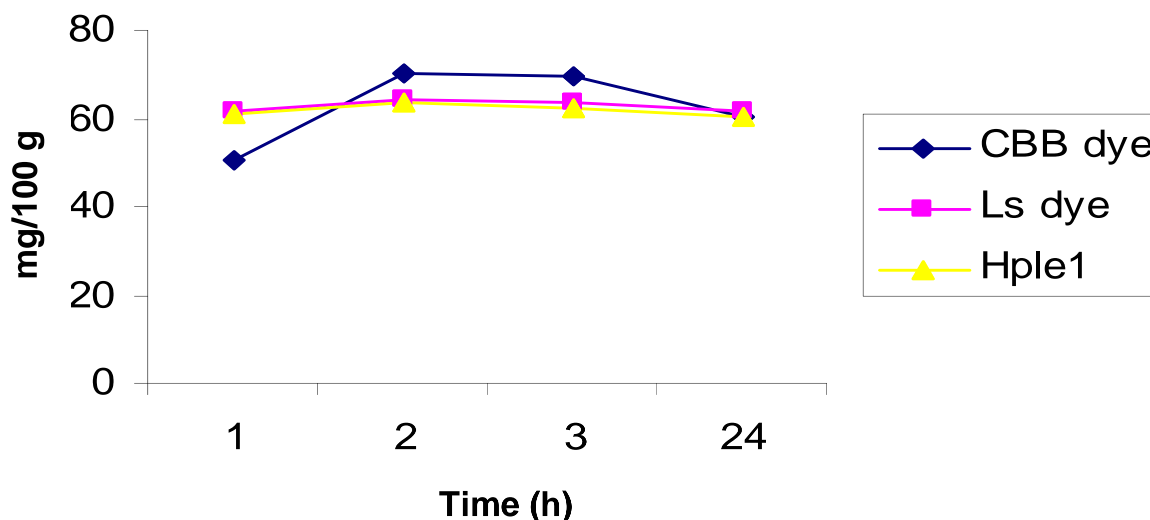

Figure 5. Comparison of staining of C.S of gram (G-3) with CBB dye, Ls dye and hple1 for different time intervals at room temperature.

means of three staining dyes (SAS Institute Software).

DISCUSSION

Gram, used as food, is a good source of protein (Jain et al., 2006; Sawhney and Randhir, 2006). Much research work has been done on this plant (Deepti et al., 2005) but in this present research work, protein of stem has been determined at three different stages of growth by Lowry's and Bradford quantitative methods. The results showed that in the case of G-1, Lowry's method gave the highest protein contents than Bradford method and also in case of G-2 Lowry method gave more protein than Bradford method but in case of G-3 both methods gave approximately equal quantities of Protein (Pandey et al., 2006). It was also observed that plant nitrogenous compounds level became high as the plant grew. Nitrogen moved from leaves to the seed and nitrogenous compound such as protein will be in high concentration, same results were determined by this study, in both plants, at first stages it gave less protein but at the third stage it gave more protein. The result is consistent with research work done by (Schiltz et al., 2005; Vision et al., 2003).

The Microscope technique is successfully used in the field of computational biology/bioinformatics (Shotton and Wiley, 1993). In this research work, light microscope was used and intensity of stained image was analyzed because each pixel of image having different intensities of color (Lee et al., 2009). In order to differentiate between different parts of the same plant or between other plants and to see the structure and function in relationship anatomical study is necessary. Anatomical sections are important in getting different and more precise information about the plant structure and this can only be obtained through microscopic study (Stephens and Allan, 2003). The combination of microscope and computer has been applied in many research works. To use computer is not only for saving time or money but it is used for accurate and reliable measurement of different compounds in biological samples. These computer tools are widely used in the medical science especially, for diagnosis purpose (Eggert and Mitchison, 2006; Noble et al., 2005; Zapletal et al., 2004; Pandey, 2002; Durant et al., 1994b; Castleman, 1998). The present research educates the use of computer in the field of research. The present work shows the comparison of colorimetric and computer software methods, the amount of protein concentration at different stages of stem growth of Gram. Computer software analysis was used by De Matos et al. (2006). He used it for the assessment of immuno-chemistry of samples by manual and computer software. The anatomical sections were stained with CBB dye, Ls dye and Hple1 for determination of protein through computer software development by the author. The data so obtained was compared with results assessed by Colorimetric methods. The results showed that the Lawsone dye extracted (Hple1) or pure form (Ls) showed same concentration by the computer software and Colorimetric determination. The high concentration of protein with CBB dye may be attributed to the color of the dye which has more intensity as compared to light orange color of Lawsone dye. This gives the idea that for computer based studies, use light dyes is preferable. The concentration of protein at different stages of deve-lopment was assessed and it was found that the increase in concentration is due to plant growth. During the plant growth, the concentration is increased as has been shown by Schiltz et al. (2005) and other authors Vision et

al. (2003).

Conclusion

Results obtained, using Lowry's and Bradford methods showed both plants having maximum amount of proteins in their third stages. The staining period of dye was also established, the staining of sections (stem of Gram) with all three dyes for 2 h gave maximum amount of protein as compared to 1, 3 and 24 h of staining periods. The results of computer software showed more concentration of protein content compared to Colorimetric methods in visible regions. The results from the computer method for two dyes are different and this may be attributed to the attachment of sites of dye and protein in different dyes. Coomassie Brilliant Blue dye (CBB dye) is attached with many sites of proteins so protein binding of CBB dye is more as compared to Lawsone dye. In the future, with this method, one can see the migration, destination and distribution of different compounds from root to the top of the plant.

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