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Haemocyte Count in Beauveria Bassiana Infection and Subsequent Oral Treatment of Ethanolic Plant Extract on 3rd Day of Fifth Instar Larvae of PM And CSR2 B. Mori L.

J. A. Chavan[1], S. S. Mohite[1] and G. P. Bhawane[2]
[1]Department of Zoology, Rajaram College, Kolhapur
[2]Department of Zoology, Shivaji University, Kolhapur

Abstract: *Haemocytes play very important role in immunological status of an insect. The significance of haemolymph and haemocyte in the various life processes and protection mechanism from pathogen, the present work was carried out the day-to-day changes in Total Haemocyte Count (THC) and Differential Haemocyte Count (DHC) in fifth instar larvae of Bombyx mori L. inoculated with Beauveria bassiana and treatment of ethanolic plant extracts on 3rd day of 5th instar larvae. Sigificance enhancement of THC in inoculated group was observed on third day and then decreased the count day by day. The plant extracts of Curcuma longa, Argimonemexicana and Clerodendrummultiflorum enhance the defence response probably by enhancing the cellular, immunity, which is actively participate in the elimination of invading pathogen like B. bassiana in PM and CSR2 race, due to which when the inoculated larvae with B. bassiana and subsequently treated with plant extracts reduce the disease incidence due to the increase THC and DHC which is helped for silkworm to eliminate the invaded microbes and help for survival. Due to elimination of pathogen by plant extract treatment silkworm crop saved and sometimes it shows more or less similar results in control groups.*

Keywords: Bombyx mori, Beauveria bassiana, Plant Extracts, Haemocyte Count.

I. INTRODUCTION

Silkworm, Bombyx mori L. is an important lepidopteron insect of economic importance that has been under domestication from 5,000 years now. Silk is "the sovereign of strands" since it is a smooth, sparkling, astonishing and exceptional fibre spun by silkworms. Successful silk production and quality attributes depend on the larval health growth and the required environmental conditions. Growth and development affect the productivity, which depends on the intricate physiological processes. In sericulture, the productivity and quality generally rely upon the rearing of disease-free healthy silkworm larvae. As such, success of sericulture depends on proper management and protection of silkworm crop from diseases Iqra Rafiq et. al., (2020). Insect blood or haemolymph is better defined as the circulating intracellular fluid filling the cavity of the body or haemocoel while bathing different tissues (Jones, 1979). A thin permeable membrane lining the haemocoel physically separates the haemolymph from direct contact with the body tissues (Ashhurst, 1979).

Insect haemolymph contains haemocytes that are suspended in the plasma. Haemocytes play multiple important roles during insect growth and development. Five types of hemocytes have been identified in the silkworm (B.mori) viz., prohemocyte, plasmatocyte, granulocyte, spherulocyte, and oenocytoid. Haemocytes have basic parts in different physiological activities (Wigglesworth, 1959). Haemocytes in the haemolymph of the insects assume a significant function in the protection mechanism. Similarly, haemolymph plays an important role in the inherent immunity response, which is induced when bacteria invade the body of the silkworm (Hou, et al., 2010). Insects possess an effective innate immune system against foreign microorganisms.

In addition, there is growing evidence that diverse classes of antibiotics have immunomodulatory effects, in addition to their antimicrobial activity (Pasquale and Tan, 2005). These studies further added that the useful activity of the

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antibiotics can be ascribed to enhance action of anti-infection agents which diminishes fundamentally the occurrence of flacherie and grasserie. With this background, the present investigation is important to study the influence of antibiotics on the haemocyte count and rearing performance in silkworm Bombyx mori L.

II.MATERIAL AND METHODS

Animal: For present study PM and CSR2 silkworm Bombyx mori L. larvae were used.

B. bassiana: B. bassiana culture were brought from MTCC, chandigarh, and the culture were maintained as per method of Govindon, et al., (1998).

Preparation of Plant Extracts: Plants were collected from fields of Kolhapur District, Maharashtra, India. The taxonomic identification of the plants was made with available literature (Yadav and Sardesai 2002).
Plant extracts were prepared by the method of Alade and Irobi (1993) with minor modifications suggested by Ahmad and Beg (2001). The collected plant material washed with distilled water and shade dried at room temperature. The materials were grinded to fine powder with the help of mixer grinder. Then these powdered materials were used for preparation of ethanolic extracts by using 50g powder mashed in 100 ml absolute ethanol for 72 hours. The mixture was stirred every 24 hours using a sterile glass rod. At the end of extraction, each extract was concentrated in vaccuo at 300C and stored at 40C until further use.

Haemocytes Count:
Total haemocyte count and differential haemocyte count was done by using the method of Praful (1994). The counting chamber and coverslip of haemocytometer were cleaned with alcohol and air dry. The haemolymph was collected by pricking the prologs or caudal horn with sterile lancet. A drop of haemolymph was immediately taken on a haemocytometer slide and placed a coverslip such that it covers the entire chambers on haemocytometer. The haemocytes on chamber were allowed to settle for about a minute then slide was placed on stage of light microscope and haemocytes were counted from the four corner squares.

For THC used the following formula,

THC = (No. of cells counted X 10) / Number of 1 sq. mm counted

For DHC Wright's stain was used dilution with 1:1 with phosphate buffer. In DHC take the clear and grease free slide. On the slide take the drop of haemolymph diluting fluid (2% versine solution) and then add haemolymph on it and prepared a smooth smear of haemolymph. The slide was kept for room temperature for drying. After half an hour the Wright's stain was sprayed on slide kept for 5-7 minutes then washed with distilled water then slide was dried and cleared in xylene and mount in DPX. The DHC was done by using light microscope.

III.RESULT AND DISCUSSION

The results obtained on 3rd day of fifth instar larvae in B. bassiana inoculation and subsequent treatment of ethanolic plant extracts are shown in Table No.1.

1. **Total Haemocyte Count (THC):**
   The total haemocyte count observed more in CSR2 race i.e., 11676.67 THC/mm3 than PM race 9086 THC/mm3 in control group on 3rd day of fifth instar. The inoculation of B. bassiana showed the increase of total haemocyte count in both races by 25.78% in PM and 22.09% in CSR2 race. The treatment of ethanolic plant extracts C. longa showed decreased THC by 8.07% in PM and by 0.10% in CSR2. In A. mexicana treated group the THC decreased in both races by 10.18% and by 16.23% in PM and CSR2 respectively over the control. The treatment of C. multiflorum also showed the decreased THC by 30.9% in PM race and by 9.28% in CSR2 race.
   The above results indicates that the increased THC in both races on 3rd day after the B. bassiana inoculation. The maximum increase of THC was observed in PM race than. The C. longa treated group showed decreased in THC as compared to control but the decreased percentage was less and it was the nonsignificant change. In A.

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mexicana treated group decrease of THC was observed in CSR2 race but it was less than PM race. The treatment of C. multiflorum showed decreased THC in both races but the higher decrease was observed in PM race than CSR2 race.

2. Differential haemocyte count (DHC):

I. Granulocyte:

In DHC observations the granulocyte observed more in CSR2 i.e., 32.67% than PM race which showed the20.18% in control groups. In B. bassiana inoculated group the decreased granulocyte count observed in PM by 48.7%, while the increased percentage was observed in CSR2 by 4.07% as compared to control groups. The C. longa treated group Showed the decreased percentage in both races by 36.9% and by 28.5% in PM and CSR2 race respectively as compared to their control groups. In A. mexicana treated group both races showed the decreased granulocyte count by 15.95% in PM race and 58.15% CSR2 race. The application of ethanolic plant extract of C. multiflorum showed the decreased count of granulocyte by 91.59% and 38.18% in PM and CSR2 races respectively.

The results obtained showed that the inoculation of B. bassiana responsible for decreased granulocyte count in PM and increased granulocyte count in CSR2 race as compared to control group. The treatment of ethanolic plant extracts all groups showed the decreased granulocyte count in both races on 3rd of inoculation. The maximum decrease was observed in PM race in C. multiflorum treated group.

II. Prohaemocyte:

The more prohaemocyte count was observed in PM race than CSR2 in control group i.e., 30.14% and 10.33% respectively. The inoculation of B. bassiana showed the decreased the count of prohaemocyte 23.05% and 41.9% in PM and CSR2 race respectively. The application of ethanolic C. longa extract showed the increased of prohaemocyte in PM by 65.7% while the decreased was observed in CSR2 race by 15.7% as compared to control group. In A. mexicana treated group PM race observed the increased count of prohaemocyte by 9.85% while the decreased count was observed in CSR2 by 12.48%. In the treatment of C. multiflorum plant extract the percentage of prohaemocyte observed in PM by 36.59% and in CSR2 decrease was observed by 12.4% as compared to their respective control groups.

The above results revealed that the inoculation of B. bassiana causes the reduction in prohaemocyte count in both races. The maximum decreased percentage was observed in CSR2 race than in the PM race. The application of ethanolic plant extracts after the inoculation of B. bassiana. the increased prohaemocyte percentage was observed in PM race. The maximum increased prohaemocyte observed in C. longa treated group. But the decreased haemocyte count observed in CSR2 race the maximum decreased prohaemocyte count was observed in C. longa treated group as compared to control group.

III. Plasmatocyte:

In control group CSR2 showed the higher percentage of plasmatocytei.e 4.33% than PM race i.e 2.06%. in B. bassiana inoculated group PM race showed significant increase of the plasmatocyte by 473.3% was observed while in CSR2 showed increased plasmatocyte by 69.51% as compared to control group. The treatment of ethanolic plant extract of C. longa observed the increased plasmatocyte count in PM race by 112.6% and decreased percentage was observed in CSR2 race by 7.62% as compared to control group. In A. Mexicana treated group both races observed the increased plasmatocyte count by 21.84% and 7.88% in PM and CSR2 races respectively. The treatment of C. multiflorum also observed the plasmatocyte count by 107.7% and 61.6% in PM and CSR2 race respectively as compared to control.

The above results indicate that the increased percentage of plasmatocyte observed in all the group including B. bassiana inoculated group except the C. longa treated CSR2 group.

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IV. Spherulocytes:

The higher spherulocyte percentage observed in CSR2 race (15.67%) than the PM race (8.19%) in the control group. The decreased spherulocyte percentage observed in both races after the B. bassiana inoculation, which was by 85.71% in PM race and by 53.4% in CSR2 race. The treatment of ethanolic extract of C. longa showed decreased spherulocyte by 49.4% in PM race and by 10.9% in CSR2 race. In A. mexicana treated group increased spherulocyte percentage observed in both the races by 253.6% and by 5.80% in PM and CSR2 race respectively. The treatment of C. multiflorum showed the increased spherulocyte in PM race and decreased in CSR2 by 84.2% and by 65.7% respectively.

The above results indicate that the decreased spherulocyte count in both races due to the inoculation of fungus B. bassiana. The percent decreased observed maximum in PM race than CSR2 race. In C. longa treated group also the decreased percentage of spherulocytes was reported. The maximum decrease was observed in PM race than CSR2. The increased or decreased percentage of spherulocyte showed the significant level as compared to control groups.

V. Adipohaemocytes:

In control, group more adipohaemocyte percentage observed in PM race than CSR2 which were 19.66% and by 16.67% respectively. The adipohaemocyte percentage increased in both races after the inoculation of fungus B. bassiana by 124.5% in PM race and 19.9% in CSR2 as compared to control group. In C. longa treated extract showed the decreased adipohaemocyte percentage by 83.8% in PM race and by 80.02% in CSR2 as compared to control group. The treatment of A. mexicana also showed the decreased percentage of adipohaemocyte by 95.2% and by 72.04% in PM and CSR2 respectively. In C. multiflorum treated group the increased adipohaemocyte percentage was observed in both races by 12.41% and by 155. 9% in PM and CSR2 race respectively.

From above results it became clear that the adipohaemocyte percentage increased in B. bassiana inoculated and C. multiflorumtreatd group in both races while the decreased percentage of adipohaemocytes observed in C. longa and A. mexicana treated group in both races as compared to control group in both races as compared to control on 3rd day of 5th instar. The maximum decreased adipohaemocyte percentage observed in A. mexicana treated larvae of PM and maximum increased was observed C. multiflorum treated group of CSR2 race when compared with control groups.

VI. Coagulocyte:

In DHC the coagulocytes observed 4.67% in CSR2 race and 3.93% coagulocytes were observed in PM race. In control group CSR2 race showed more coagulocytes percentage than PM race. The inoculation of B. bassiana showed the increased percentage of coagulocytes in CSR2 race. While no was change observed in PM race. The treatment of C. longa showed the decreased coagulocytes percentage by 29% and increased percentage by 189.5% in PM and CSR2 respectively. In A.mexicana treated group both races observed the increased coagulocytes percentage by 327.4% and 28.47% in PM and CSR2 race respectively. The treatment of C. multiflorum showed the decreased coagulocytes percentage by 43.7% in PM while no change observed in CSR2 race as compared to control group.

VII. Oenocytoid:

The more oenocytoid percentage observed in PM race was 16.29% and in CSR2 11.67% in control group. The reduced percentage of oenocytoid observed in both races after the B. bassiana inoculation. The percent decrease was 31.79% in PM and 11.48% in CSR2. The treatment of ethanolic extract of C. longa showed significant decrease oenocytoids by 53.65% and by 46.70% in PM and CSR2 respectively as compared to control. In A. mexicana treated group PM showed decreased oenocytoid by 91.83 % and increased oenocytoid in CSR2 by 34.54% percentage over control group. In C. multiflorum treatment both races showed the decreased oenocytoid count by 13.38% in PM race and by 3.77% in CSR2 race when compared with the control group 3rd day on inoculation of 5th instar larvae.

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The results on haemocyte count on 3rd day of 5th instar larvae inoculated with B. bassiana and subsequent treatment with plant extracts were reported. In the present study seven types of haemocytes were observed in silkworm Bombyx mori i.e., granulocytes (GRs), prohaemocytes (PRs), Plasmatocytes (PLs), Spherulocytes (Spls), Adipohaemocytes (Ads), Coagulocytes (COs), Oenocytes (OEs), similar observations on haemocyte types were made by earlier workers in lepidopteron insects including silkworm B. mori (Raina, 1976; Beeman et al., 1953; Ribeiro et al., 1996; Riebeiro, and Brehlin, 2006).

Inoculation of B. bassiana, causes significant increase in THC on 3rd day. The similar results reported by Mallikarjuna et al., (2002), that the THC were increased in initial stage of infection and decreased in the later stage of infection i.e., on the 6th day. These present results agreed with these findings. Once the entomopathogenic fungus have penetrated in host integument and gained access to the nutrient rich haemocoel, they are confronted with the host humoral and cellular defence mechanism (Butt et al., 1988, Butt and Humber, 1989, Vey and Gotz, 1986). In the control group they have reported that the THC was significantly low as compared to the treatment because the increase may represent the defence response of silkworm, A. mylitta against the invading pathogen. The observed data agreed with observations of the earlier workers. They have made investigations that once entomapathogenous fungi have penetrated in the host integument and gained access the nutrient rich haemocoel (Kirankumar et al., 2012). The cellular responses to the infections have been worked out in many insects by earlier workers (Horohav and Dunn, 1983). Similar types of results obtained when the effect of systematic fungicide studied on the haemocyte count in B. bassiana infected silkworm, B. mori.

The treatment of ethanolic plant extracts having the antifungal properties suppressing the multiplication of pathogen and boosting the immunity level, which leads the similar results with control or increased than control. In the inoculated and control, the counts of haemocyte gate increased after inoculation with CPV up to 2nd day of infection. Then there was a decrease during the period ranging from 3-8 days. In normal control THC was significantly low as compare to the treated became the increase may represents the defence response of silkworm A. mycotta against the invading pathogen.

Therefore, the observations of the present study agreed with the observations of the earlier workers. They have reported that once the entomophagous fungi penetrated the host integument and gained access to nutrient rich haemolymph. The observed data of the present study agreed with the earlier investigator that the number may increase (Balvenkatasubbiah et al., 2001; Al-Attar, 2010) and decrease to count foreign body when infected. Earlier workers (Horohov and Dunn, 1983) have worked out the cellular responses to infection in many insects.

IV. CONCLUSION

From the above study, it is clear that the plant extracts of C. longa, A. mexicana and C. multiflorum enhances the defence response probably by enhancing the cellular immunity, which is actively participated in the elimination of invading pathogen like B. bassiana in PM and CSR2 race, due to which when the inoculated larvae with B. bassiana and subsequently treated with plant products reduce the disease incidence due to the increase THC and DHC which is helped for silkworm to eliminate the invaded microbes and help for survival.

ACKNWOLEDGEMENT

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PLATE – I TYPES OF HAEMOCYTES

1: a-Plasmatocyte, b-Prohaemocyte; 2-4: Sperulocyte; 5: Plasmatocyte; 6: Adipohaemocyte; 7: Oenocytoid; 8: a-Granulocyte, b- Prohaemocyte; 9: a- Sperulocyte and Prohaemocyte; 10:Coagulocyte; 11: Granulocyte; 12: Oenocytoid

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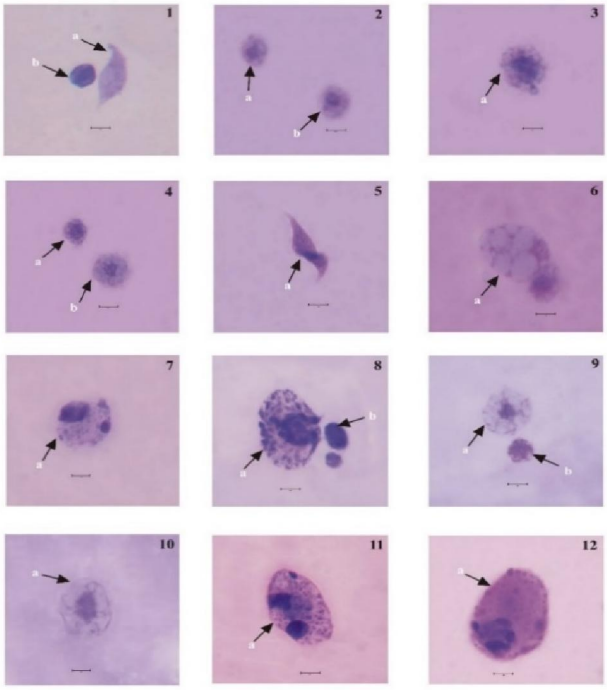

Fig. 1. Effect Of *B. Bassiana* Infection And Subsequent Oral Treatment Of Ethanolic Plant Extracts On Total Haemocyte Count (Thc)on 3rd Day Of Fifth Instar Larvae Of PM And CSR2 *B. Mori* L.

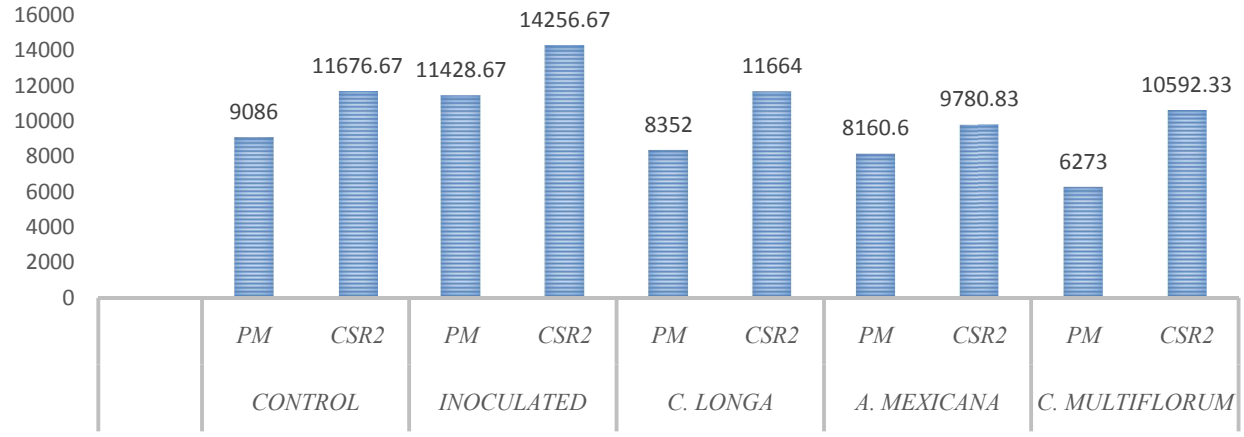

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FIG. 2. Effect of *B. bassiana* infection and subsequent oral treatment of ethanolic plant extracts on differential haemocyte count (DHC) on 3rd day of fifth instar larvae of PM and CSR2 *B. mori* L

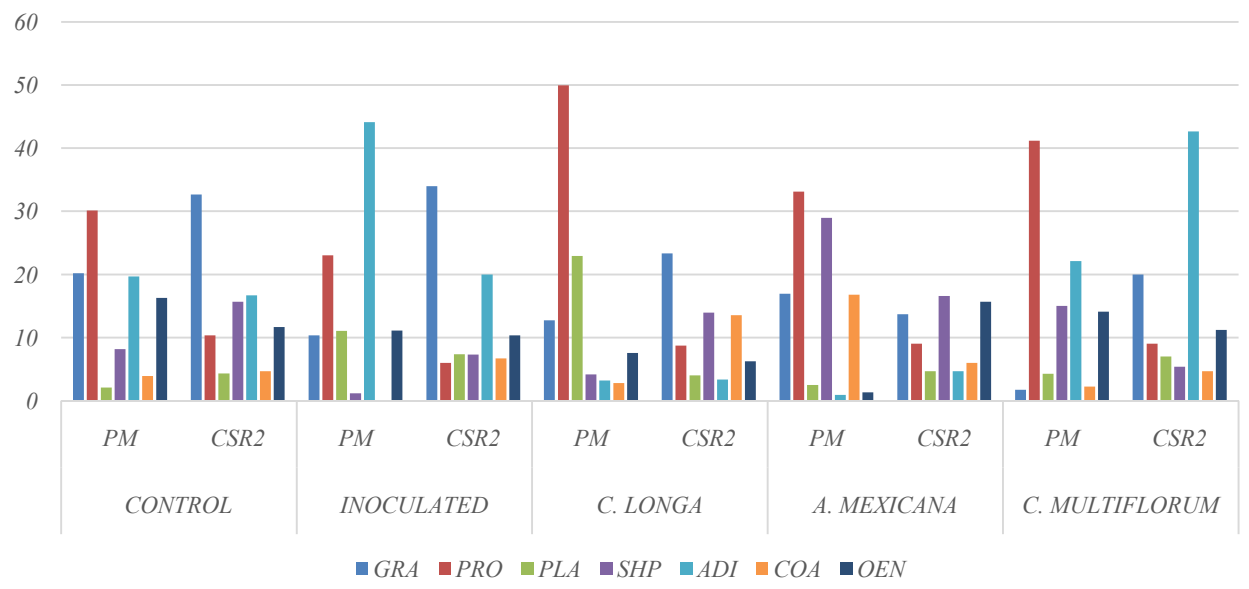

Table No. 1: Effect of B. bassiana infection and subsequent oral treatment of ethanolic plant extracts on total haemocyte count (THC) and differential haemocyte count (DHC) on 3rd day of fifth instar larvae of PM and CSR2 B. mori L.

GROUPS	RACE	THC	DHC						
			GRA	PRO	PLA	SHP	ADI	COA	OEN
CONTROL	PM	9086.00 ± 166.69	20.18 ± 1.61	30.14 ± 3.14	2.06 ± 0.95	8.19 ± 0.65	19.66 ± 0.79	3.93 ± 0.12	16.29 ± 0.34
	CSR 2	11676.67 ± 612.50	32.67 ± 3.79	10.33 ± 1.53	4.33 ± 3.21	15.67 ± 8.14	16.67 ± 7.02	4.67 ± 1.15	11.67 ± 4.51
INOCULATED	PM	11428.67 ± 43.39 **(+25.78)** ***	10.34 ± 0.98 **(-48.7)** ***	23.03 ± 0.03 **(-23.05)** **	11.07 ± 0.07 **(+473.3)** ***	1.17 ± 0.31 **(-85.71)** ***	44.15 ± 0.51 **(+124.5)** ***	0.00 ± (0.00)	11.11 ± 0.00 **(-31.79)** **
	CSR 2	14256.67 ± 371.18 **(+22.09)** ***	34.00 ± 19.16 **(+4.07)** *	6.00 ± 4.36 **(-41.9)** **	7.34 ± 4.05 **(+69.51)** **	7.29 ± 5.03 **(-53.4)** **	20.00 ± 4.00 **(+19.9)** **	6.67 ± 0.58 **(+42.82)** **	10.33 ± 1.53 **(-11.48)** **NS**
C. LONGA	PM	8352.00 ± 102.53 **(-8.07)** NS	12.72 ± 1.06 **(-36.9)** **	49.96 ± 0.96 **(+65.7)** **	22.92 ± 0.66 **(+112.6)** ***	4.14 ± 0.64 **(-49.4)** **	3.17 ± 0.23 **(-83.8)** ***	2.79 ± 0.26 **(-29.00)** *	7.55 ± 0.09 **(-53.65)** ***
	CSR 2	11664.00 ± 202.06 **(-0.10)** NS	23.33 ± 2.52 **(-28.5)** ***	8.70 ± 3.10 **(-15.7)** *	4.00 ± 0.61 **(-7.62)** NS	13.96 ± 5.13 **(-10.9)** **	3.33 ± 2.31 **(-80.02)** ***	13.52 ± 2.01 **(+189.5)** ***	6.22 ± 0.38 **(-46.70)** ***
A. MEXICANA	PM	8160.6 ± 115.70 **(-10.18)** **	16.96 ± 0.52 **(-15.95)** **	33.11 ± 2.11 **(+9.85)** *	2.51 ± 1.52 **(+21.84)** NS	28.96 ± 3.86 **(+253.6)** ***	0.93 ± 0.06 **(-95.2)** ***	16.80 ± 0.25 **(+327.4)** ***	1.33 ± 0.57 **(-91.83)** ***

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	CSR 2	9780.83 ± 296.83 **(-16.23)** **	13.67 ± 4.73 **(-58.15)** ***	9.04 ± 2.06 **(-12.48)** NS	4.67 ± 2.89 **(+7.85)** NS	16.59 ± 9.42 **(+5.80)** NS	4.66 ± 2.18 **(-72.04)** ***	6.00 ± 1.73 **(+28.47)** **	15.67 ± 3.21 **(+34.57) NS**
C. MULTIFLORUM	**PM**	6273.00 ± 139.19 **(-30.9)** *	1.70 ± 0.53 **(-91.59)** ***	41.17 ± 1.17 **(+36.59)** **	4.28 ± 0.60 **(+107.7)** NS	15.0 ± 1.36 **(+84.2)** ***	22.10 ± 0.20 **(+12.41)** **	2.21 ± 0.18 **(-43.7)** **	14.11± 0.30 **(-13.38) NS**
	CSR 2	10592.33± 347.43 **(-9.28)** NS	20.00 ± 3.00 **(-38.18)** **	9.04 ± 2.60 **(-12.4)** NS	7.00 ± 3.61 **(+61.6)** **	5.37 ± 2.84 **(-65.7)** **	42.67 ± 4.51 **(+155.9)** ***	4.67 ± 5.51 **(0.00) NS**	11.23 ± 1.27 **(-3.77) NS**

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