

Persistence of the transformed *Paenibacillus polymyxa* expressing CRY1C in the plant leaves and its effect on chlorophyll and carotenoid

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Abstract: The transformed *Paenibacillus polymyxa* bacterial strain expressing Cry1C (tNMO10) that has dual function: bio-fertilizer and biocontrol agent of the Lepidopteron insects was used in spraying the cotton plants. plant leaves were checked for the presence of the transformed bacteria inside it by PCR detection of *cry1C* gene and total protein profiling. Two pairs of specific primers that give 2.2 kb and 3.7 kb amplified products from *cry1C* gene were used. Moreover, chlorophyll A, B & total chlorophyll A+B and carotenoid were determined. The data revealed, the presence of Cry1C toxin protein in the total cellular protein pattern of sprayed plant leaves with tNMO10 while was not found in those sprayed with parent strain (NMO10) and the reference strain *Bt aizawai*. The PCR could detect the 2.2 kb and 3.7 kb of the *cry1C* gene with plant leaves that sprayed with the tNMO10. Chlorophyll A, B and A+B showed increase in concentration in the second and fourth week after planting in the plant leaves treated with tNMO10 over the concentration of that leaves treated with the NMO10. The plant leaves that not treated with bacteria (control) showed the lowest concentration. The values of CA, CB and CA+B in the second week in plant leaves that treated with control, untransformed strain NMO10 and transformed strain tNMO10 respectively were as follow: CA: 18.63< 24.24< 28.59 mg/L, CB: 14.49< 21.73< 23.88 mg/L, CAB: 33.45< 41.65< 47.36 mg/L. In addition, the carotenoid content showed the same behavior of chlorophyll giving the following values in the second week with control, untransformed strain and transformed strain respectively, 4.18< 5.19< 5.69. Thus, the transformed bacteria tNMO10 caused improvement in the cotton plants through the increase of chlorophyll and carotenoid contents due to its presence survival and expressing its toxin Cry1C protein inside the cotton leaves. [Nahed Abdel Ghaffar A. Ibrahim; Amal Ibrahim Hussien; Adel El-Sayed Hatem; Hani K. Aldebis and Enrique Vargas-Osuna. **Persistence of the transformed *paenibacillus polymyxa* expressing CRY1C in the plant leaves and its effect on chlorophyll and carotenoid.** *Life Sci J* 2014;11(8):433-442]. (ISSN:1097-8135). <http://www.lifesciencesite.com>. 57

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1. Introduction

Recent survey recorded that Egyptian soil become poor in organic matter, the content of total soluble nitrogen in the soil ranged between 0.07-0.11 and 13-30 ppm which it is very low values (Sanaa, 2010). Losses of nitrogen fertilizers are not only impact to the environment but also are great economic losses.

Nitrogen supply has large effect on leaf growth because it increases the leaf area of plant and, on that way, it influences on photosynthesis. photosynthetic proteins represent a large percentage of total leaf content of nitrogen (Evans, 1983; Field and Mooney, 1986; Evans, 1989) and chlorophyll content is proportional to leaf nitrogen content (), also carotenoid have essential role in photosynthesis. So that, Leaves exhibit a structural and functional adaptation of the photosynthetic apparatus to the light intensity

experienced during their growth (Prioul *et al.*, 1980). Nitrogen is a structural element of chlorophyll and protein molecules, then affects formation of chloroplast and accumulation of chlorophyll in them (Daughtry, 2000; Amalotis *et al.*, 2004).

Chlorophyll in the process of photosynthesis sign for physiological responses of plant to nitrogen fertilization (Marija *et al.*, 2011). Leaf chlorophyll content differ within high limits (from 0.05 to 30% of fresh weight matter), the ratio of chlorophyll A to B is 3:1. These ratio differ according to environmental factors affecting the plant and to the physiological process of plant. The highest chlorophyll limits in plant are found in the flowering phase. Chlorophyll playing a role in organogenesis process (Simova *et al.*, 2001). Nitrogen is part of the enzymes associated with chlorophyll synthesis (Chpman and Barreto, 1995) and the chlorophyll concentration represent, a relationship

of crop nitrogen content and yield level (Blackmer and Scheperts, 1995)

The plasmid PHTNC3 containing *cry1C* gene (Nahed *et al.*, 2008). This plasmid was used to transform the *Paenibacillus polymyxa* bacterial strain NMO10 (Nahed and Omar, 2009). NMO10 bacterial strain was isolated from rhizosphere of cotton plants from Egyptian soil (Feibo and Omar, 1998), it had high efficiency in fixing nitrogen and high ability to colonize the phyllosphere. The transformed bacterial strain tNMO10, was resulted from the transformation process and showed high activity against Lepidopteron insect, *Spodoptera littoralis*, as well, its ability to fix nitrogen increased. In a study on the tNMO10 compared with the non transformed original strain NMO10 and with *Bacillus thuringiensis* (*Bt*) var aizawai by (Amal *et al.*, 2011), they reported that the transformed bacteria tNMO10 was more toxic (LC50 = 7.04×10^7 spores + crystals/ ml) than *Bt* aizawai (LC50 = 8.47×10^7 spores + crystals/ ml). Moreover, the tNMO10 showed high levels of persistence into foliar tissues and insecticide activity were found at least until 30 days after foliar treatment. On the other hand, its application in the plant increased the amount of nitrogen up to (52.93 mg/plant) as compared with the non transformed strain NMO10 (28.79 mg/plant) and improved soil nitrogenase activity (370.39 Mol C₂H₂/gm/hr) as compared with NMO10 (300.34 Mol C₂H₂/gm/hr) when using ARA (acetylene reduction assay). Thus, the aim of the present study, is to complete studies on the transformed bacterial strain tNMO10 through determining the impact of treatment of cotton plant leaves with different doses of the transformed *Paenibacillus polymyxa* bacterial strain tNMO10 on yield parameters including presence of toxin in plant leaves and its effect on chlorophyll and carotenoid.

2. Material and Methods

Bacterial strains :

Paenibacillus polymyxa NMO10 (nitrogen fixing bacteria) and its transformed form tNMO10 (NMO10 expressing *cry1C* gene) were obtained from Dr Nahed A. A. Ibrahim, microbial molecular biology (MMB) lab, MMB dept., AGERI, ARC, Giza, Egypt. *Bacillus thuringiensis* strain aizawai was used as a reference strain which naturally expresses the *cry1C* gene.

1. Detection of bacteria in the cotton leaf tissues:

1.1 *Cry 1C* detection by PCR

Two pairs of specific primers were used for detection of *cry1C* gene. 1AF&1AR (Regev *et al.*, 1996) that give 2.2 kb PCR product from *cry1C* gene. 1AF (ACG GAG GAT CCA TAT GGA GGA TCA AAA TAA AAA TC) and 1AR (CTC TTG TAT GGG GAT CCT AAC ATT AAG CTT TTA TC). The other pair was 1AF & 1CR (Nahed *et al.*, 2008), 1CR (TTA TTC CAT GAG TAA AAG CTC TTC C) that give

3.7 kb the full length of *cry1C*. PCR were done according to the protocol of (Ceron *et al.*, 1995) where 10 ul of the juice of the grinded leaves were added to 90 ul of sterilized water to prepare the DNA template. The reaction conditions were according (Regev *et al.*, 1996).

1.2 *Cry1C* toxin protein detection by total cellular protein profiling from leaf tissues: according (Laemmli, 1970) total protein patterns were done from both bacterial cells and from leaves of cotton plants. *P. polymyxa* NMO10, transformed *P. polymyxa* tNMO10 and *Bt* var aizawai were grown from the original inoculums in 5ml LB liquid medium (Tryptone 10 g/L, yeast 5 g/L, sodium chloride 5 g/L) to get culture in vegetative cells and other time they were grown in 5 ml T3 medium (tryptone 3 g/L, tryptose 2 g/L, yeast extract 1.5 g/L, sodium phosphate buffer 50 mM, MnCl₂ 0.005 g/L, in 1 L dH₂O) to get culture in spore cells. cotton plants (maintained in a special green house for genetically modified microorganisms) were treated by spraying the leaves with spore suspension of MNO10, spores-crystals culture of tNMO10 and spores-crystals culture of *Bt* aizawai equivalent to LC 90 concentration. The leaves were taken 14 days after treatment for check for *Cry1C*. Prior of working on the leaves, they were superficially disinfected with 10 % sodium hypochlorite for 10 minute, then, washed three times with sterile distilled water. They were finally crushed in liquid nitrogen using a sterilized mortar and stored at - 20 C° for use.

2. Chlorophyll and carotenoid contents:

The method described by (Hiscox and Israelsten, 1979) was followed. 100 mg of the sample (small pieces of leaves) were introduced into test tube containing 7 ml dimethyl sulfoxide (DMSO) and incubated for overnight to extract chlorophyll and carotenoid, then the extract was filtered and transferred to a cylinder and made up to 10 ml volume with DMSO. For determining chlorophyll; the absorbance was measured by spectrophotometer at 640 nm and 662 nm and for carotenoid the absorbance was measured at 470 nm. The chlorophyll A content (CA), chlorophyll B (CB) and the total chlorophyll (CAB) were calculated according the (Arnon, 1949) equation and the carotenoid were calculated according (villanueva *et al.*, 1985) equation.

Arnon equation:

$$C A = 12.7 \times (OD_{662}) - 2.69 \times (OD_{644}) \text{ mg / L}$$

$$C B = 22.9 \times (OD_{644}) - 2.69 \times (OD_{662}) \text{ mg / L}$$

$$C AB = 20.2 \times (OD_{644}) + 8.02 \times (OD_{662}) \text{ mg / L}$$

Villanueva equation:

$$\text{Carotenoid} = [A_{470} - 1.28 (CA) + 56.7 (CB)] / (256 \times 0.906)$$

3. Statistical analysis

Data relating to the values in the tests development, are subjected to analysis of variance. In

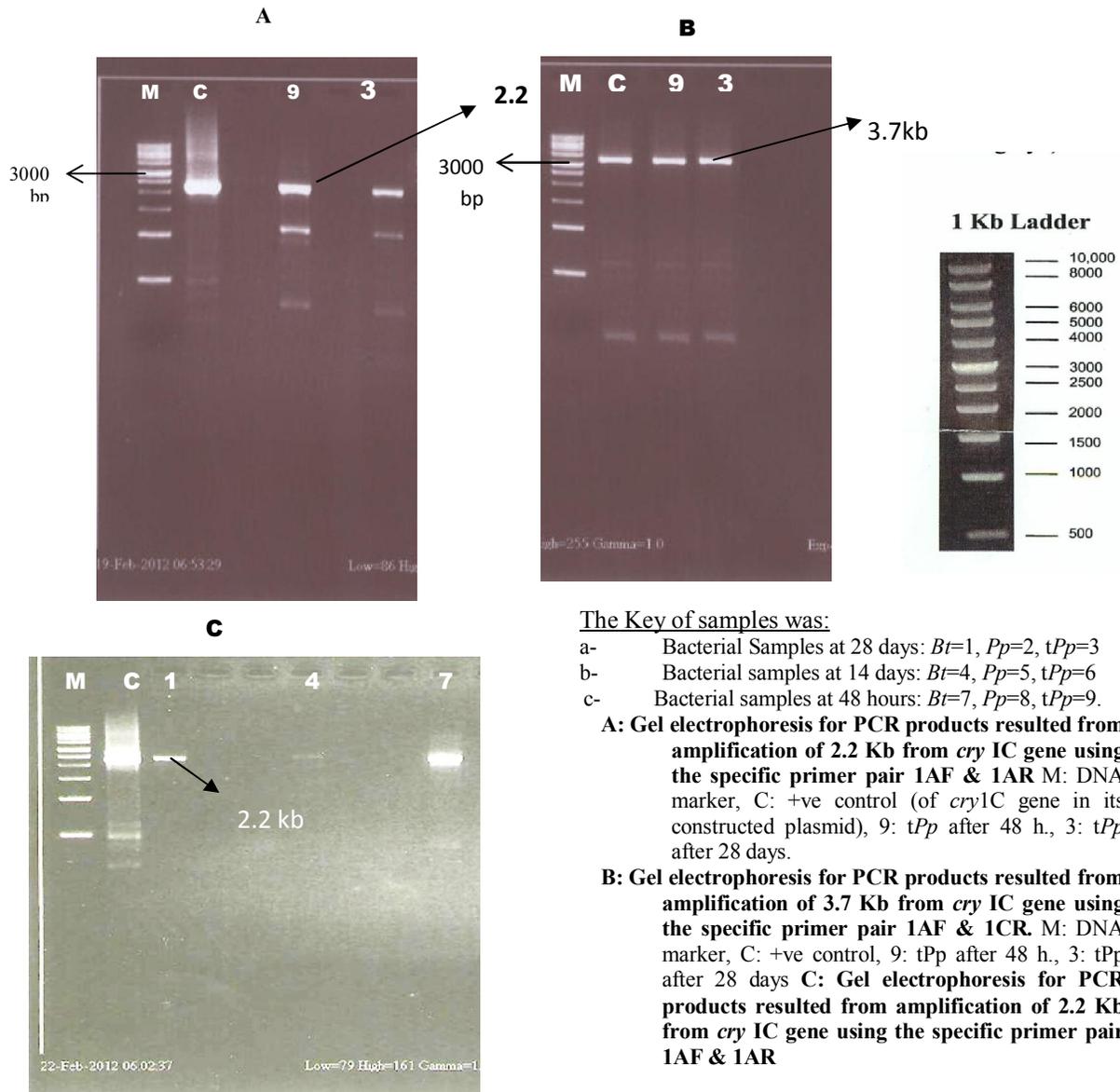
case there are significant differences, means were compared to test minimum difference significant (MDS) at the 5% significance level. All analysis were performed using the statistix 8.0 program.

3. Results

Cry 1C analysis

The treated cotton leaves with the transformed *P.p.* bacterial strain tNMO10, parent strain NMO10 and the reference bacterial strain *Bt aizawai* were used for detection of *cry1C* gene. The treated cotton plant leaves were collected at 2, 14 and 28 days after treatment. The detection of *cry1C* in the

treated leaves by specific PCR reaction using 1AF&1AR and 1AF&1CR primers showed the PCR products (2.2 kb) & (3.7 kb) respectively corresponding to the *cry1C* (fig 1). Fig 1. A showed the 2.2kb from cotton leaves treated with tNMO10 at 2 and 28 days. Fig 1.B showed the 3.7 kb from the same cotton leaves. Fig 1.C showed the 2.2 kb of *cry1C* from treated leaves with *Bt* reference strain at 2, 14, and 28 days. On the other hand, no any PCR products detected with cotton plant leaves that treated with non transformed parent strain NMO10.



The Key of samples was:

- a- Bacterial Samples at 28 days: *Bt*=1, *Pp*=2, *tPp*=3
- b- Bacterial samples at 14 days: *Bt*=4, *Pp*=5, *tPp*=6
- c- Bacterial samples at 48 hours: *Bt*=7, *Pp*=8, *tPp*=9.

A: Gel electrophoresis for PCR products resulted from amplification of 2.2 Kb from *cry1C* gene using the specific primer pair 1AF & 1AR M: DNA marker, C: +ve control (of *cry1C* gene in its constructed plasmid), 9: *tPp* after 48 h., 3: *tPp* after 28 days.

B: Gel electrophoresis for PCR products resulted from amplification of 3.7 Kb from *cry1C* gene using the specific primer pair 1AF & 1CR. M: DNA marker, C: +ve control, 9: *tPp* after 48 h., 3: *tPp* after 28 days

C: Gel electrophoresis for PCR products resulted from amplification of 2.2 Kb from *cry1C* gene using the specific primer pair 1AF & 1AR

M: DNA marker, C: +ve control, 1: *Bt aizawai* after 28 days, 4: *Bt* at 14 days, 7: *Bt* at 48 h

Figure 1. The detection of *cry1C* in the treated leaves by specific PCR reaction using 1AF&1AR and 1AF&1CR primers showed the PCR products (2.2 kb) & (3.7 kb) respectively corresponding to the *cry1C*

Protein analysis

The protein profiles of the transformed bacteria *P. polymyxa* tNMO10 respects the untransformed parent strain NMO10 and *Bt* var aizawai. The band corresponding to the Cry1C toxin at the molecular mass 135 KDa is detected only in the spore cells in the *Bt* aizawai as transformed *P. polymyxa* tNMO10 (Fig. 2). Protein profiles obtained from cotton leaf samples that were treated 14 days earlier with tNMO10, NMO10, and *Bt* aizawai. One can observe the presence of the band corresponding to Cry1C only in the treated leaves with tNMO10 (Fig.3).

Levels of chlorophyll and carotenoid

In figure 4, the indicated values of chlorophyll A for four weeks, in cotton plants treated with non-transformed *P. polymyxa* and *P. polymyxa* transformed. Significant differences between treatments ($p < 0.0001$) were found between weeks ($p < 0.0001$). The amount of chlorophyll was higher than the control to the second week after treatment with *P. polymyxa* transformed and Untransformed *P. polymyxa* until the third week. Finally, in the fourth week values were reduced to below baseline.

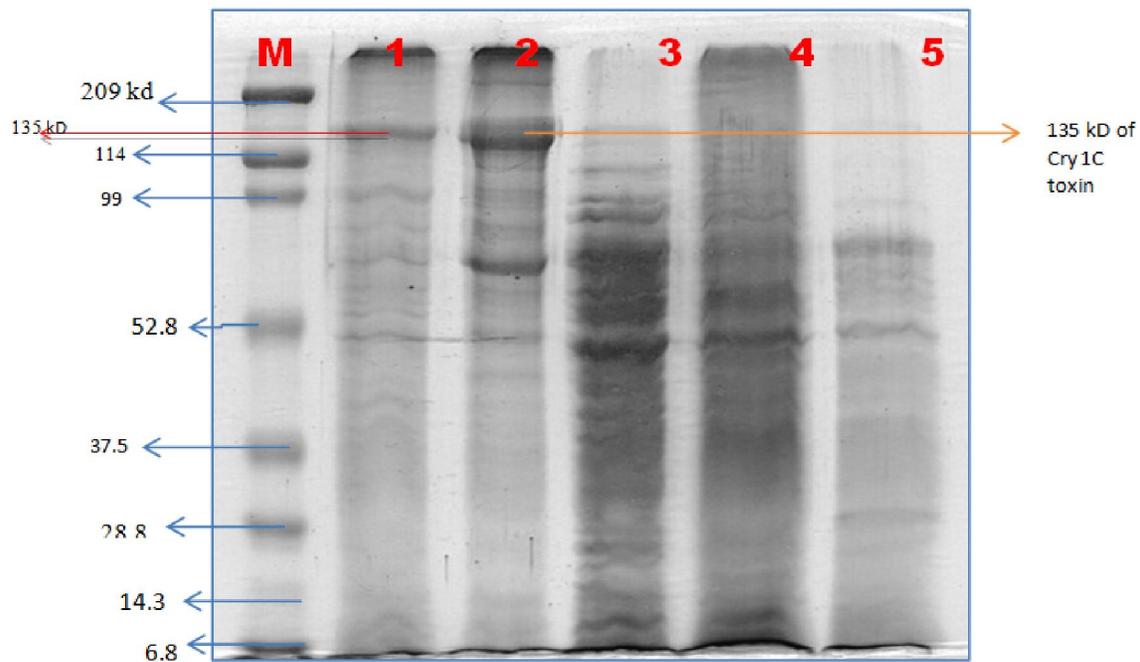


Fig 2: SDS-PAGE for protein profiling of bacterial cells of transformed *Pp* compared to that from parent cells *Pp* and bacteria *Bacillus thuringiensis Bt aizawai*

M: protein marker and its bands has been written on left side of the photo

- 1: protein from *Bt* bacterial cells
- 2: protein from spore form of t*Pp*
- 3: protein from vegetative form of t*Pp*
- 4: protein from spore form of parent strain *Pp*
- 5: protein from vegetative form of *Pp*

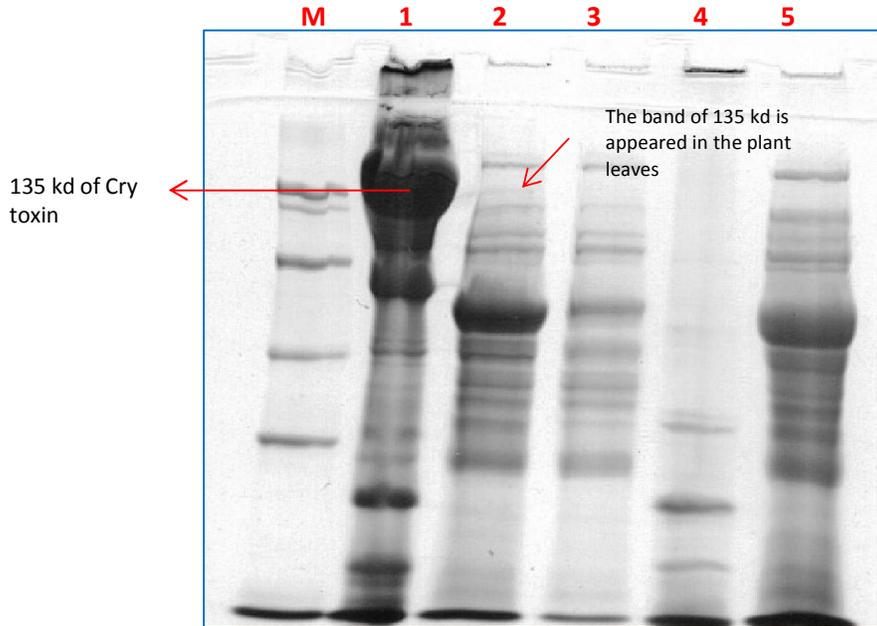


Fig 3: SDS-PAGE of protein pattern from plant leaves and bacterial cells
 M: protein marker
 1: Total cellular protein from bacterial cells of *tPp*
 2: total cellular protein from plant leaves sprayed with *tPp*
 3: total cellular protein from plant leaves that not treated with bacteria (control)
 4: total cellular protein from parent bacterial strain *Pp*
 5: total cellular protein from plant leaves sprayed with *Pp*

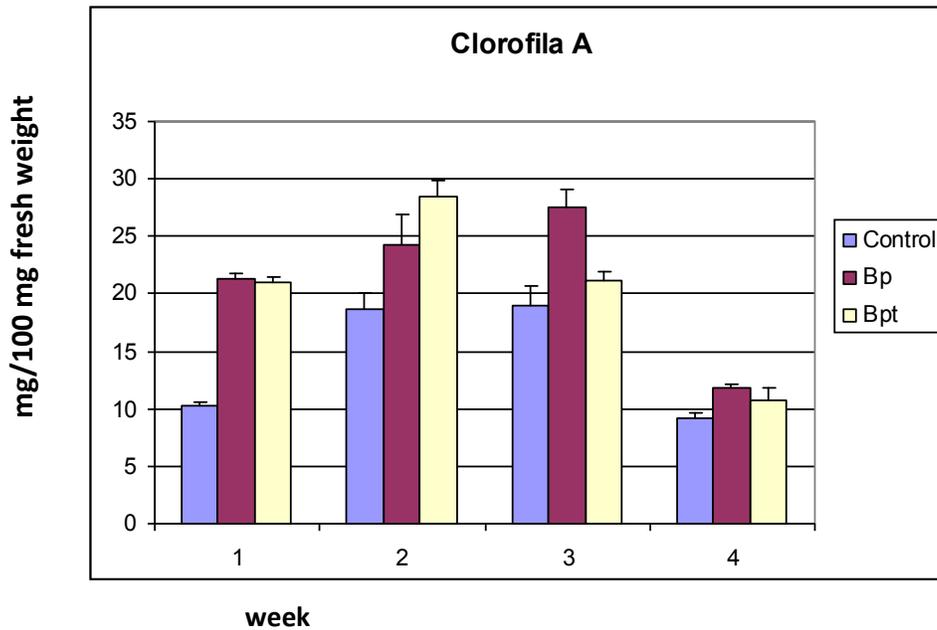


Figure 4: Chlorophyll A levels over the four weeks in cotton plants treated with untransformed *Paenibacillus polymyxa* (*Pp*) and transform *Paenibacillus polymyxa* (*tPp*)

Analysis of Variance Table for CLA (chlorophyll A)

Source	DF	SS	MS	F	P
Treat	2	340.98	170.492	37.32	0.0000
week	3	976.66	325.555	71.27	0.0000
Treat*week	6	171.96	28.660	6.27	0.0005
Error	24	109.63	4.568		
Total	35	1599.23			

Grand Mean 18.611; CV 11.48

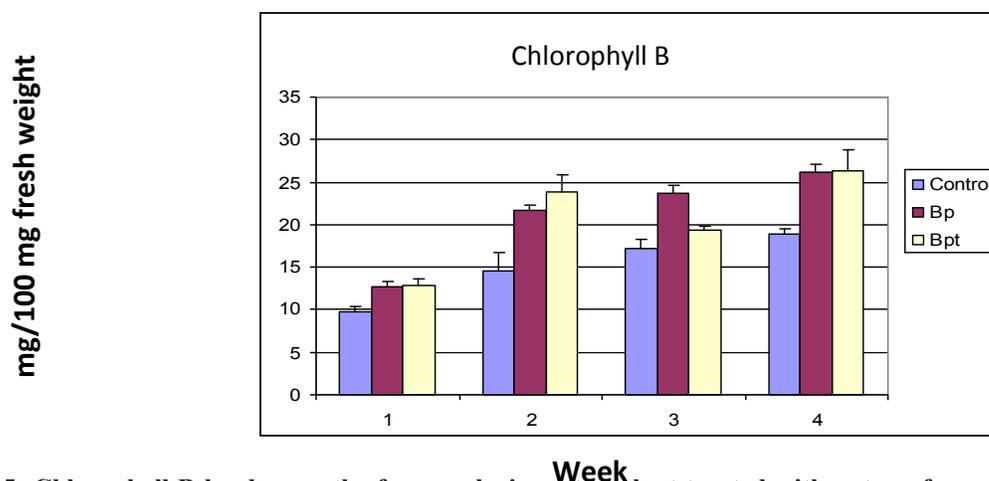


Figure 5: Chlorophyll B levels over the four weeks in cotton plant treated with untransformed *Paenibacillus polymyxa* (NMO10) and transformed *Paenibacillus polymyxa* (tNMO10).

Analysis of Variance Table for CLB (chlorophyll B)

Source	DF	SS	MS	F	P
Treat	2	266.33	133.163	26.03	0.0000
week	3	699.21	233.071	45.56	0.0000
Treat*week	6	71.64	11.939	2.33	0.0644
Error	24	122.76	5.115		
Total	35	1159.94			

Grand Mean 18.926; CV 11.95

LSD All-Pairwise Comparisons Test of CLB (Chlorophyll B) for Treatments

Treat	Mean	Homogeneous Groups
2	21.077	A
3	20.613	A
1	15.089	B

Alpha 0.05; Standard Error for Comparison 0.9233
 Critical T Value 2.064; Critical Value for Comparison 1.9057
 Error term used: Error, 24 DF

LSD All-Pairwise Comparisons Test of CLB (Chlorophyll B) for Weeks

Week	Mean	Homogeneous Groups
4	23.838	A
3	20.053	B
2	20.034	B
1	11.780	C

Alpha 0.05; Standard Error for Comparison 1.0662
 Critical T Value 2.064; Critical Value for Comparison 2.2005
 Error term used: Error, 24 DF

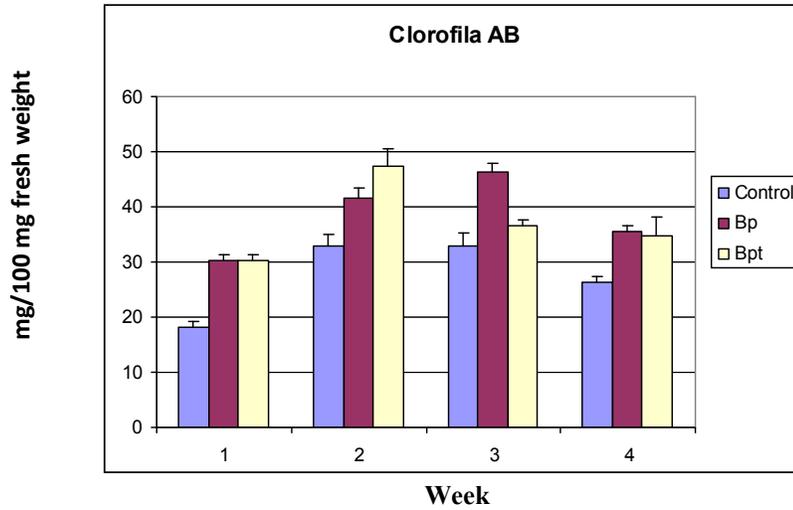


Figure 6: Chlorophyll levels A + B during the four weeks in cotton plants treated with untransformed *Paenibacillus polymyxa* (Pp) and transformed *Paenibacillus polymyxa* (tPp)

Analysis of Variance Table for CLAB (Chlorophyll A+B)

Source	DF	SS	MS	F	P
Treat	2	12.5551	6.2775	26.42	0.0000
Week	3	43.7819	14.5940	61.43	0.0000
Treat*week	6	2.9213	0.4869	2.05	0.0979
Error	24	5.7015	0.2376		
Total	35	64.9598			

Grand Mean 4.5606; CV 10.69

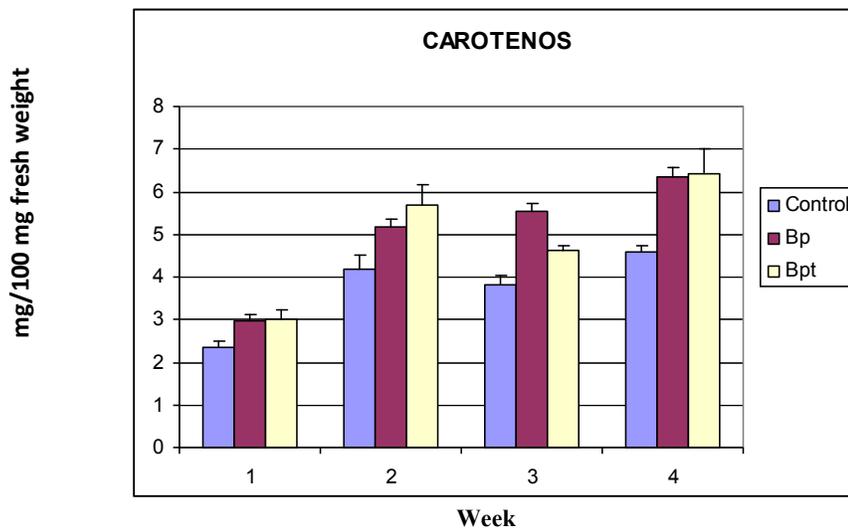


Figure 7: Carotenoid levels during the four weeks in cotton plants treated with untransformed *Paenibacillus polymyxa* (Pp) and transformed *Paenibacillus polymyxa* (tPp).

Analysis of Variance Table for CAROTENOIDS

Source	DF	SS	MS	F	P
Treat	2	12.5551	6.2775	26.42	0.0000
Week	3	43.7819	14.5940	61.43	0.0000
Treat*week	6	2.9213	0.4869	2.05	0.0979
Error	24	5.7015	0.2376		
Total	35	64.9598			

Grand Mean 4.5606; CV 10.69

LSD All-Pairwise Comparisons Test of
CAROTENOIDS for Treatment

Treat	Mean	Homogeneous Groups
2	5.0175	A
3	4.9375	A
1	3.7267	B

Alpha 0.05; Standard Error for Comparison 0.199

Critical T Value 2.064; Critical Value for Comparison 0.4107

Error term used: Error, 24 DF

LSD All-Pairwise Comparisons Test of
CAROTENOIDS for Weeks

Week	Mean	Homogeneous Group
4	5.7811	A
2	5.0200	B
3	4.6567	B
1	2.7844	C

Alpha 0.05; Standard Error for Comparison 0.2298

Critical T Value 2.064; Critical Value for Comparison 0.4742

Error term used: Error, 24 DF

Figure 5 shows the indicated values of chlorophyll B during four weeks in cotton plants treated with non-transformed *P. polymyxa* and *P. polymyxa* transformed. Chlorophyll B values differ significantly between treatments ($p < 0.0001$) and between weeks ($p < 0.0001$). From the first week the chlorophyll B content was higher in both treatments compared to the control. In the second week chlorophyll B values significantly increased, in the plants treated with *P. polymyxa* transformed and that treated with non-transformed *P. polymyxa*. In the third week a significant decrease only obtained in the plants treated with transformed *P. polymyxa*, and recover in the fourth week. In figure 6 levels of chlorophyll A + B are shown for the four weeks in cotton plants treated with non-transformed *P. polymyxa* and *P. polymyxa* transformed. The values differed between treatments ($p < 0.0001$) and between weeks ($p < 0.0001$). The pattern in the variation of the content was similar to that proposed for the chlorophyll A content. In Figure 7 the listed values of carotenoid during four weeks in cotton plants treated with non-transformed *P. polymyxa*, *P. polymyxa* transformed. the Statistical analysis showed significant differences between treatment ($p < 0.0001$). Since the first week, values in *P. polymyxa* treated plants were greater than those of control. In general, the values were growing in the course of four weeks, with significant increase in the second week compared to the first in all treatments. As with the results obtained with the chlorophyll B, the amount of carotenoid was reduced significantly only in the third week for treatment with *P. polymyxa* transformed to recover their levels in the fourth week.

4. Discussion

PCR detection of *cry1C* gene in the treated cotton plant leaves with *P. polymyxa* untransformed NMO10, *P. polymyxa* transformed tNMO10 and *Bt aizawai*, showed the presence of *cry1C* in both the tNMO10 and *Bt aizawai* up to 28 day of planting. On the other hand, protein profiling of the cotton leaves that treated with those bacterial strains, showed the Cry1C toxin protein only in the treated plant leaves with transformed *Paenibacillus polymyxa* tNMO10, but not in the plant leaves that treated with *Bt aizawai*. These results describe the higher activity of the treated plant leaves with transformed *P. polymyxa* against Lepidopteron insects than that treated with *Bt aizawai* (Amal *et al.*, 2011).

Moreover, Inoculation of *P. polymyxa* (both the original and the transformed) produced an increase chlorophyll levels that were maintained above untreated plants for the first two weeks. For chlorophyll B, AB and carotenoid was an increasing trend during the four weeks of the trial, but in the third week there was a decrease only in the treatment with *P. polymyxa* transformed. These results agree with the increase in chlorophyll levels chickpea plants inoculated with *P. polymyxa* (Akhtar and Siddiqui, 2007). In a review by (Natheer *et al.*, 2013; Munees and Kibret, 2014) reported the role of plant growth promoting rhizobacteria (PGPR) including *Paenibacillus polymyxa* in improving fertilization, nitrogen fixation, increasing chlorophyll A and B content and improving the yield of the crops.

Biljana and Stojanovic, (2005) who did their study on chlorophyll and carotenoid content in wheat cultivars. They found that the carotenoid content in the leaf showed the greatest values but these values were lower than values of chlorophyll. The data of our study showed the same results which the chlorophyll increased with the same rate as carotenoid but not with the same levels. In the second week after planting the content of chlorophyll of cotton plants that treated with control, non transformed bacterial strain NMO10 and transformed bacterial strain tNMO10 were CA: 18.63 < 24.24 < 28.59 mg/L, CB: 14.49 < 21.73 < 23.88 mg/L, CAB: 33.45 < 41.65 < 47.36 mg/L respectively and the content of carotenoid were 4.18 < 5.19 < 5.69 respectively. In a study by (Sanaa, 2010), the treatment of cotton plant leaves with kinetin and GA3 (Gebrajic acid) significantly increased leaves content of chlorophyll A, B, A+B and carotenoid due to the treatment of plants with organic matter, i.e, increase in nitrogen content. Biljana and Aca, (2009), proved in their study on different wheat cultivars a very close link between chlorophyll and nitrogen content in the leaf. A study by (Marija *et al.*, 2011) on lemp plants, proved, leaves with higher nitrogen concentration

showed higher chlorophyll content as compared to not fertilized plant control. It is increased with the increase of fertilization dose in different treatments. This study also agree with the present study in that the chlorophyll content increased with the experimental period and from day 29 to day 43. Our study showed in the second week, high level of chlorophyll in samples treated with tNMO10 while decreased in the third week and re in height again in the fourth week. Marija *et al.* (2011), found that additional nitrogen fertilization significantly increased chlorophyll content seven days after the first treatment. Thus, these leaf chlorophyll content can be an indication of tissue nitrogen concentration. Saied and Maryam, (2011), proved in their study on effects of N fertilizer on chlorophyll content in three cultivars of Maize that chlorophyll significantly increased with increasing of nitrogen levels. In a study by (Pramanik and Bera, 2013), they reported that the total chlorophyll content of different growth stages of rice plant was significantly increased for the transplanting of 10-days seedling. These data agree with ours in which the chlorophyll content increased from the first week, then, highly increased in the second week. Continuously, in the same study, 30- day old seedlings gave the lowest value for total chlorophyll content. These results also, agree with our results in which the chlorophyll contents declined down in the third week and re increased in the fourth week but not in the same levels of the second week. Kumar *et al.* (1995), (Abdel Wahab, 1998; Verma *et al.*, 2004) recorded that N content in the third leaf and chlorophyll A content, increased with increasing nitrogen. In a study by (Lin and Ehleringer, 1982) on cultivars of papaya, they reported that after 15 days of development, the photosynthetic rate began a constant decline, and they also mentioned a lot of references that recorded the same observation. the study by (Lin and Ehleringer, 1982), also recorded the observation, that, the higher the initial maximum photosynthetic rate the greater was the rate of decrease and associated with decrease of photosynthetic rate consequently, decreases in the chlorophyll content. Mauromicale *et al.* (2006), that their study was on potato crop to assess chlorophyll fluorescence parameters in response to nitrogen dose. A positive linear relationship between nitrogen supply and chlorophyll content was obtained. These all previous results are interpreted that the increase of nitrogen associated directly with increased chlorophyll resulting in increased photosynthesis process. Thus, from the literatures and the obtained data of the present work, the treatment of the cotton plants with the bacterial strain *Paenibacillus polymyxa* NMO10 and its transformed one tNMO10 increased the nitrogen content (Amal *et al.*, 2011), consequently, increased the chlorophyll and carotenoid contents (this

work). Moreover, the transformed *P.p.* tNMO10 strain showed more increase of the nitrogen, chlorophyll and carotenoid as a result of bearing the Cry1C protein as proved by PCR and protein profiling. So, the tNMO10 improved the yield of cotton plant as well as its ability to make the cotton plants protected against Lepidopteron insects due to the presence of Cry1C toxin inside the plant leaves (this work).

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