Use of the bioagent Bacillus subtilis to break dormancy of buds in table grape, Flame seedless, under organic farming condition

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ABSTRACT--Table grape is considered one of the most important fruit crops, either for local consumption, or for exportation. In subtropical countries such as Egypt, there is no adequate winter chilling to stimulate bud burst. Farmers, especially those aim to export their grape fruits, familiarized to use chemical treatment with Dormex (hydrogen cyanamide 2-5%) to break bud dormancy. According to European organic regulation EC. 834/2007 and 889/2008, Annex I and II dormex is not allowed. The present work offers biological method that could be replaced to Dormex in organic farms. *Bacillus subtilis* (three isolates) were selected from micro flora of dormant buds according to their efficacy in cellulose decomposition. Selected purified and identified *B.subtilis* isolates were used to determine their effect on grape bud brust compared with Dormex 2%. Data obtained showed that using these bacterial isolates either as single isolate or as a mixture contains two isolates at different concentrations led to significant increase in number of burst buds compared with control treatment (water). Data also showed that positive correlation between concentrations of the bacterial cell /ml and efficacy of the treatment was detected. Data also indicated that the different isolates as single or as a mixture varied in their effect. In this respect, the mixture of $B_2 + B_3$ (60 X 10^6 c.f.u) caused the highest effect whereas the lowest effect was noticed when *B. subtilis* isolate B_2 was used alone at concentration of 20 X 10^6 c.f.u / ml. Moreover, using of bacterial suspension as spray resulted in low effect compared with using the same suspension as brushing using soft brush. Repeating treatment every week for 2 or 3 times led to increase of the number of burst buds compared with using only one application

Key words: Table grape, *Bacillus subtilis.* bud burst, organic farming, break dormancy .

1 INTRODUCTION

Table Grape is considered as one of the most important fruits in Egypt. Table grape is produced in Egypt for local consumption and also for exportation. According to Ministry of Agriculture, the total area of table grape was about 192.934 feddan in year of 2014, which yield about 1.596169 ton. The total exported table grape reached about 88.144 ton during 2013 with value of about 183.35 USA Dollars. European market only receive Egyptian grape till 15 June. Farmers familiarized to use Dormex (hydrogen cyanamide) to break buds dormancy, to export their table grape before June 15 to get considerable high price. In organic agriculture, farmers cannot use Dormex according to European organic regulation Eu. 834 / 2007 and 889/ 2008. Many attempts were carried out to use natural materials to replace the chemical method (Dormex) with natural product. **Abd El Moniem and Abd-Allah (2008)** studied the effect of green algae *Chlorella vulgaris* cells extract as foliar spray on bud burst, growth and yield of Superior grape vines. Application of algal extract at 25 to 100% had the higher percentage of bud burst and fruiting buds. They added that 50% concentration of algae extract is suggested to be beneficial from economical view and slight promotion was detected on fruit quality as a result of using algal extract above 50%, which led to increase TSS, TSS/ acid ratio and total sugars and decreasing total acidity rather than control. **Maldonado *et al.* (2010)** studied the effect of the mixture of garlic compounds (GC) compared with Dormex on bud break dormancy and on cluster quality in four table grape cultivars. Quality of fruits of all treated cultivars showed excellent results. Cluster weight and berry sizes were larger than the other treatment (Dormex).They concluded that, ability of (GC) to break dormancy in table grape could be used for table grape produced under organic farming in hot regions. **Sabry *et al.* (2011)** used jasmine oil as a breaking bud dormancy agent for Flame seedless grape vines. They used jasmine oil at three concentrations (0.1, 0.2 or 0.3%), which were sprayed alone or in combination with 3 and 5% Dormex. The best results were obtained in the combination of 0.2% jasmine oil with Dormex 3% compared with control treatment 5% Dormex.

Ahmed *et al.* (2014) examined ten plant extracts namely turmeric at 5 %, cinnamon at 10%, ginger at 10%, colocynth at 5%, nigella at 5%, olive oil at 5%, clove at 5%, garlic at 5%, red chilies at 5%, and coffee at 10% as well as four chemical agents namely H2O2 at 10%, salicylic acid at 5 to 10%; thiourea at 2 to 8% and Dormex at 1 to 6 % to break dormancy of grape buds. This study gives evidence that it can use plant extracts as coffee, red chilies, garlic and clove as natural safety substances to break bud dormancy.

2 Materials and Methods

In all experiments, unless otherwise indicated, all laboratory work was carried out at central laboratory of organic agriculture. Grape buds, used for isolation different microorganisms, were collected from farms located in 6 October city and Behera Governorate. All field work was done during season 2014 at grape orchard cv. (Flame seedless), at Behera governorate . The used farm has sandy loam soil with 8.1 pH, and about 1500 ppm salts, whereas irrigation water contains about 1200 ppm salts.

Isolation, purification and identification of the used microorganisms:

Samples of dormant grape buds were collected from different grape vines during December (2011). One gram of each sample was placed in bottle contain 99 ml of sterilized water. Bottles contain buds and sterilized water were kept on shaker for 2 hrs. to, mechanically release any microorganisms established on dead scales of the dormant buds. Serial dilution plate technique (Johnson *et al*.,1959) was used (to isolate *Bacillus* spp. on nutrient agar medium (Oedjijono and Dragar,1993).

Inoculated Petri dishes were incubated at 30 ±1 ^{0}C. All incubated dishes were examined periodically, every day, to detect the developed colonies on medium surface. Different developed colonies were purified and then, pure isolates were kept in tubs contain NGA medium for identification. Pure isolates were identified according to **Schleifer (2009).**

Screening of some *B.subtilis* isolates on cellulose decomposition:

Ten pure identified bacterial isolates were screened for their efficacy on cellulose decomposition using medium contained Carboxy Methyl Cellulose (CMC) as a sole source of carbon. Plates contain CMC, were inoculated, at plate's center, with a loop full of bacterial suspension. Inoculated plates were kept under room temperature. After 2 days all plates were treated using solution of Congo red (1% w/v) for 15 minutes. followed by 1.0 M Na Cl for 15 minutes, according to method developed by **El-Sersy *et al.* (2010)** to detect clear zone surround bacterial growth. The highest clear zone refer to the highest capacity of the isolate to cellulose degradation. Diameter of clear zones developed around the bacterial colony were determined. The most effective three bacterial isolates were selected for further studies.

Field experiments
Inocula preparation:

The most effective identified bacterial isolates (*Bacillus subtilis* B_1, B_2 and B_3) were used to inoculate bottles contained 100 ml of N.G. Broth. Inoculated bottles were incubated at 28 ^{o} C for 3 days. *B. subtilis* cultures were shacked and then number of colony forming units (c.f.u) was adjusted to be $60X10^6$ using sterilized saline solution 0.1%. Different adjusted bacterial cultures were formulated as suspensions contain desired number of c.f.u. plus 5% liquid soap, to reduce water tension, in addition to 1% arabic gum solution to increase viscosity and adhesive capacity for suspension. Different formulated adjusted bacterial suspensions were used to treat grape dormant buds as spray treatment (spraying) or by cover buds with formulated suspension using smooth brush (brushing). These bacterial preparations were used to carry out following experiments, under field condition.

Evaluation of efficacy spraying *B. subtilis* isolates as single or in mixture form, at different concentration, on grape bud burst :

Flame seedless grape vines, two years old, were selected. Grape vines were divided into 21 groups, each group contained 5 trees. Different bacterial treatments were distributed randomly using complete randomize plots design, to evaluate efficacy of different isolates. Different bacterial treatments , *i.e.* B_1, B_2, B_3 or the mixture of B_1+ B_2, B_1+B_3 and B_2+ B_3 at three different concentrations (20, 30 and $60X10^6$ c.f.u/ ml water with total 18 treatment were used. Three control treatments , *i.e*. saline solution 0.1, Dormex 2% and material used to formulate bacterial suspension (saline 0.1+ 5% liquid soap + 1% Arabic gum) were used. Different treatments were sprayed on grape vines, during the first week of January, using 20 L. spraying motor. Treated plants were examined every week to determine number and percentages of burst buds. After one month, early February, the total percentage of burst buds were calculated in each treatment. Data were tabulated and statistically analysis according to SAS (1996). Significance of different means were compared using Duncan's Multiple Range test (P= 0.05).

Efficacy of *B. subtilis* isolates, in single or in a mixture forms, as brushing, at different concentrations, on grape bud burst :

The same bacterial and control treatments were repeated on another group of Flame seedless trees with only one difference. Different treatments were applied to grape buds by brushing using smooth brush.

Effect of numbers of application of *B. subtilis* in single or in a mixture forms, as spray, on grape bud burst:

The same six bacterial formula in addition to the previously mentioned three check treatments were used as spray treatment. Treated grape vines were divided into three groups (30 trees for each). One group received the treatment only once, the second received the treatment for two times with seven days interval, where as the third group received three applications with seven days interval. Treated vines were examined periodically and data recorded and statistically analyzed as describe before.

Effect of numbers of application of *B. subtilis* in single or in a mixture forms, as brushing, on grape bud burst

The previous experiments were repeated on another group grape of vines with only one difference, all treatment were applied as brushing instead of spraying in the previous experiments.

3 Results and Discussion

Efficacy of some *B.subtilis* isolates on cellulose decomposition:

Data presented in Table (1) indicate that all *B. subtilis* isolates, show digesting action for CMC compare with control treatment. *B. subtilis* isolates varied in their reaction to CMC break down expressed as a clear zone around bacterial growth (Johnsen and Krause, 2014). The highest effect was noticed for isolate B_1 where 30 mm clear zone was recorded, followed by isolate B_3 then isolate B_2 , being 25 and 22 mm clear zone , respectively. The lowest effect was detected with isolate B_6 , being 3 mm clear zone. No effect was occurred by isolate B_7. According to these results B_1, B_2 and B_3 were selected for further studies. This variation may be due to the variation in capacity of different isolates in celluletic enzyme production.

Efficacy of spraying *B. subtilis* isolates as single or a mixture form on grape bud burst :

Table (2) indicates that, a clear significant differences between Dormex treatment and any other treatment were detected. Data also indicate that all *B. subtilis* isolates were significantly increased the percentages of bud burst compared with control (2) and control (3) treatments. Clear variations among the effect of bacterial isolates each alone or in mixture, regarding their effects on break dormancy and bud burst, were detected. In addition, the mixture of B_2+ B_3 at concentration of 60 X 10^6 c.f.u/ ml. gave the highest percentage of bud burst compared with the other bacterial treatments and controls (2) and (3). When *B. subtilis* isolate (B_2) was used at concentration of 20 X 10^6 c.f.u/ /ml the lowest percentage of bud burst was recorded. These results could be explained in the light of fact that these bacterial isolates have capacity to digest dead celluletic scales that covered dormant buds. By removing this scales, this help bud to burst (Ahmed *et al.*, 2014).

Table 1: Screening the efficacy of some *B.subtilis* isolates on cellulose decomposition using carboxymethyl cellulose (CMC).

B. subtilis isolates	Diameter of clear zone(mm)
1	30
2	22
3	25
4	15
5	5
6	3
7	0
8	7
9	19
10	18
Control	0

Table 2: Evaluation the efficacy of spraying *B. subtilis* isolates as single or in a mixture form, at different concentrations, on Flame seedless grape bud burst.

Concentrations / Bioagents	20X10⁶/ml.	30X10⁶/ml.	60X10⁶/ml.
B. subtilis 1	42.5 k*	51.2 gh	54.6 ef
B. subtilis 2	30.1 n	38.5 l	48.7 hij
B. subtilis 3	45.6 jk	50.2 hi	54.8 ef
B. subtilis 1 + *B. subtilis* 2	34.3 m	47.8 ij	51.1 gh
B. subtilis 1 + *B. subtilis* 3	54.2 fg	61.8 d	69.7 c
B. subtilis 2+ *B. subtilis* 3	57.6 e	68.9 c	77.8 b
Dormex (control 1)	89.2 a	89.2 a	89.2 a
Formula alone (control 2)	20.4 o	20.4 o	20.4 o
Water (control 3)	0.0 p	0.0 p	0.0 p

LSD at 0.05= 3.22

*Values with same letter are not significantly different.

Efficacy of brushing *B. subtilis* isolates in single or in a mixture form on grape bud burst:

Results shown in Table (3) show that using a mixture containd B^{2}+ B^{3} at concentration of 60x 10^{6}c.f.u./ ml. resulted in the highest effect on grape bud burst compared with the other bacterial treatments and the controls (2) and (3). Dormex at concentration of 2% show slight significant effect , which recorded 89.2% compare with 78.9% in case of the mixture of (B_2+ B_3). Data also show that clear positive correlation between increase bacterial cell concentrations in the used brushing material and the efficacy of the treatment was noticed. When different isolates compared with each other, the mixture of B_2+ B3 was the best, where as the isolate B_2 showed the lowest effect and only 49.8% was recorded when this preparation was used to brush buds at the concentration 60X 10^{6} c.f.u./ml. These results could be explained in the light of work of El-Sayed (2013), she indicated that use of *B. subtilis* on strawberry fruits led to increase reduced sugars. Increase reduced sugar in buds lead to accelerate bud burst as mention by Ahmed *et al.* (2014). From formerly discussed Tables (2 and 3), it could concluded that in all cases, using the bacterial preparation as brushing give better results than use the same bacterial preparation as spraying. This because brushing deliver the bacterial cells to all buds in homogeneous way.

Table 3: Evaluation the efficacy of brushing *B. subtilis* isolates in single or in a mixture form, at different concentrations, on Flame seedless grape bud burst.

Concentrations / Bioagents	20X10⁶ c.f.u /ml	30X10⁶ c.f.u /ml	60X10⁶ c.f.u /ml
B. subtilis 1	47.3 j*	55.3 gh	56.6 fg
B. subtilis 2	37.8 l	40.3 kl	49.8 ij
B. subtilis 3	42.3 k	52.8 hi	56.1 fgh
B. subtilis 1 + *B. subtilis* 2	43.1 k	50.0 ij	54.6 gh
B. subtilis 1 + *B. subtilis* 3	59.3 f	66.7 d	72.3 c
B. subtilis 2+ *B. subtilis* 3	63.1 e	72.3 c	78.9 b
Dormex (control 1)	89.2 a	89.2 a	89.2 a
Formula alone (control 2)	20.4 m	20.4 m	20.4 m
Water (control 3)	0.0 n	0.0 n	0.0 n

LSD at 0.05= 3.46

*Values with same letter are not significantly different.

Effect of number of applications of *B. subtilis* in single or in a mixture forms as spray or brushing on grape bud burst:

Data in Tables (4 and 5) reveal that using different bacterial isolates as spray or brushing for one or two or three times led to clear significant differences compared with control (2) and (3) treatments. Dormex at 2% for one time, showed the highest effect and 89.2% bud burst was recorded at early February with no significant difference when compared with the mixture of B_2+ B_3. Data also indicate that using any bacterial treatment as brushing for three times gave highest percentage of bud burst compare with using the same treatment as spray. Data also show that clear positive correlation between number of applications, either, as spraying or brushing, and efficacy of treatment. Data in these tables indicate that the highest effect was achieved when the mixture of B_2+ B_3 as brushing for three times was used, being 89.6% bud burst. This result very close to the effect of Dormex 2% where 89.2% was recorded, using Dormex or bacteria result in decomposing scales on dormant buds, which allow buds to be burst. Increasing the number of application lead to increase enzyme activates which help in accelerating decomposition of these scales (El-Sayed, 2013). According to these facts we can understand mode of action of Dormex and *B. subtilis* isolates on bud burst.

Table 4: Effect of number of applications of *B. subtilis* in single or in a mixture forms, as spray on Flame seedless grape bud burst.

No. of applications / Bioagents	1	2	3
B. subtilis 1	51.2 gh*	54.1 f	61.3 e
B. subtilis 2	38.5 j	49.2 hi	54.1 f
B. subtilis 3	54.8 f	53.2 fg	59.1 e
B. subtilis 1 + *B. subtilis* 2	47.8 i	52.6 fg	53.6 fg
B. subtilis 1 + *B. subtilis* 3	61.8 e	67.8 d	74.5 c
B. subtilis 2+ *B. subtilis* 3	68.9 d	75.5 c	86.3 b
Dormex (control 1)	89.2 a	89.2 a	89.2 a
Formula alone (control 2)	20.4 k	20.4 k	20.4 k
Water (control 3)	0.0 l	0.0 l	0.0 l

LSD at 0.05= 2.89

*Values with same letter are not significantly different.

Table 5: Effect of numbers of application of *B. subtilis* in single or in a mixture forms, as brushing, on Flame seedless grape bud burst.

No. of applications / Bioagents	1	2	3
B. subtilis 1	55.3 fg*	58.0 f	63.6 de
B. subtilis 2	40.3 i	52.5 gh	55.2 fg
B. subtilis 3	52.8 gh	55.6 fg	62.1 e
B. subtilis 1 + *B. subtilis* 2	50.0 h	51.6 h	57.6 f
B. subtilis 1 + *B. subtilis* 3	66.7 d	73.1 c	78.8 b
B. subtilis 2+ *B. subtilis* 3	72.3 c	80.3 b	89.6 a
Dormex (control 1)	89.2 a	89.2 a	89.2 a
Formula alone(control 2)	20.4 j	20.4 j	20.4 j
Water (control 3)	0.0 k	0.0 k	0.0 k

LSD at 0.05= 3.33

*Values with same letter are not significantly different.

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