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转基因 *Bt* 棉对根际土壤微生物群落的影响

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摘要:根际箱中种植2个转基因 *Bt* 棉品系99BC-4,99BC-8及其非 *Bt* 受体泗棉3(SM3)后,于花期采集其根际土壤。BIOLOG检测显示,整个168 h的实验室培养期间,根际土壤微生物群落的每孔颜色平均变化率(AWCD)随着时间的推移呈现出差异性的S型曲线;AWCD值显示,GP2板低于GN2板和ECO板,意味着供试土壤内革兰氏阳性菌对碳源的利用能力较低。功能多样性分析表明,99BC-8和SM3根际土壤微生物群落的丰富度高、多样性强,集中了较多的主导微生物种,因而表现出高代谢能力和功能多样性。PCA得出,GN2板前2个主成分分别占总变量的35.59%和19.37%,GP2板前2个主成分分别占30.57%和18.89%。推理认为,Bt毒蛋白可能刺激了某些微生物利用优势碳源以促其生长,主导着土壤微生物的反应,虽然在2个转基因 *Bt* 棉品系根际土壤中其表现不一致。

关键词:转基因 *Bt* 棉;Biolog微生物鉴定系统;土壤;微生物群落;主成分分析

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Effects of transgenic *Bt* cotton on rhizospheric soil microbial communities

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Abstract: After cultivation of 2 transgenic *Bt* cotton lines (99BC-4, 99BC-8) and their non-*Bt* recipient Simian3 (SM3) in rhizoboxes, the rhizospheric soils during blooming period were sampled. Biolog characterization revealed that during the whole laboratory incubation for 168 h, *AWCDs* of the rhizospheric soil microbial communities exhibited differential sigmoidals of C substrate utilization with incubation time, and the soils showed lower *AWCDs* in GP2 plate than in GN2 plate and EcoPlate, meaning that the G^+ bacteria in the soils possessed a weaker C source metabolic ability. Functional diversity analysis indicated that the rhizospheric soils of 99BC-8 and SM3 had a larger richness and diversity of, and centered more dominant species in the microbial communities, reflecting a high metabolic potential and functional diversity. Principal Component Analysis (PCA) identified that the first 2 PCs accounted respectively for 35.59%, 19.37% in GN2 plate, and 30.57%, 18.89% in GP2 plate of total variation. It can be deduced that Bt endotoxin likely stimulated the growth of some specific microbes by using the preferential C sources, which likely dominated the soil microbial responses, though inconsistent in 2 transgenic *Bt* lines.

Key words: Transgenic *Bt* cotton; Biolog; Soil; Microbial community; PCA

1 Introduction

Transgenic *Bt* cottons, into which the *Cry* gene encoding insecticidal Cry1Ac Bt toxic protein was introduced, have been grown worldwide for their resistance to cotton bollworm (e.g. *Helicoverpa armigera*)[1-2]. However, along with the increasing potential for widespread commercial use and the potential benefits of transgenic crops, more and more concerns have been triggered about their potentially ecological and environmental risks[3-7]. Although risk assessments have been extensively conducted, whether or not

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the risk imposed on soil environment has occurred still remains controversial[3-6]. Here the effects of transgenic *Bt* cotton on rhizospheric soil microbial communities are reported after cultivation in China.

2 Materials and Methods

2.1 Rhizobox design and soil sampling and processing

Trial site, cotton variety, rhizobox used for cultivation and soil sampling & processing were referred as [8]. Each sample was immediately put in a sterile plastic bag and transported to the laboratory in a cooled box, then sieved (2 mm) and homogenized in a rotary cylinder, and finally, stored at 4 ℃ before used for Biolog characterization.

2.2 Biolog characterization

Biolog method was used by direct incubation of soil suspensions in GP2, GN2 MicroPlates and EcoPlate (Biolog Inc., Hayward, Calif.), containing respectively 95, 95 and 31 different C sources in individual wells to determine changes in the utilization rate of individual substrates. Each fresh sample (10 g) was suspended in 100 mL saline solution (0.9% NaCl), reciprocally shaken at room temperature for 30 min at 120 rpm. After centrifugation at 1 000 g for 10 min, the pellet was suspended in 100 mL above-mentioned saline solution. Each suspension (5 mL) was used to make a 10-fold dilution series up to 10^{-3}, from which 150 μL of supernatant was inoculated into each well of GP2, GN2 plates and EcoPlate. One well without C source in each plate was used as control well. The plates were incubated in the dark at 25 ℃ in a sealed polyethylene bag and read at 590 nm at start and then every 12 h for 7 d using ELISA plate reader.

2.3 Statistical analysis

All the experiments were performed at least 3 repetitive independent treatments. An analysis of variance was carried out by using the SPSS13 software. The values are expressed as means ± SD. The significant differences among the means were calculated by using LSD-test. Statistical significance threshold was set to less than 0.05 for *p* value.

The Average Well Color Development (AWCD) method proposed by Garland and Mills[9] was used for analysis of the Biolog data. *AWCD* at a particular time was calculated using the following equation.

$$AWCD = \Sigma(Ci - R)/n$$

where *Ci* is OD for the *i* – th well with C source and *R* is that for the control well without C source. *n* means the number of C sources contained in each plate, i. e., 95, 95 and 31 respectively. The number of positive wells in each plate is described as *S* (richness). The functional diversity indices of microbial communities were referred from [10 – 12]. The indices concerned were calculated from the data at 72 h of incubation when even the slowly-growing microbes could utilize the C sources in the wells and since for the more slowly utilized C sources, longer incubation times would be required to examine the full extent of C source us. Shannon diversity index was calculated using the following equation.

$$H' = -\Sigma\, p_i \ln p_i$$

where $p_i = n_i / \Sigma n_i$, $n_i = Ci - R$ while McIntosh diversity index calculated as

$$U = \sqrt{(\sum n_i^2)}\ .$$

And Shannon evenness index was calculated as

$SE = H' / \ln S$ while McIntosh evenness index calculated as

$$ME = \frac{N - U}{N - N/\sqrt{S}}$$

where $N = \Sigma n_i$. Also, Simpson index illustrated as

$$1/D = 1/\sum \frac{n_i(n_i - 1))}{N(N-1)}$$

and Gini index as follows,

$$G = 1 - \frac{\sum_{i=1}^{w}\sum_{j=1}^{w}|n_i - n_j|}{2w^2 AWCD}$$

, where *w* is total number of all OD values.

Principal Components Analysis (PCA) of normalized data to elucidate the pattern of variation of soil microbial community profile was also performed using the SPSS 13 software. The plot of the PC scores for samples was used to display differences in metabolic diversity patterns. Relationships among samples were obtained by plotting scores of their first 2 PCs in 2 dimen-

sions, that is, samples with similar *AWCD* data sets were located close to one another while dissimilar data sets far apart.

3 Results

3.1 AWCD variation of soil microbial communities

AWCDs in GP2, GN2 plates and EcoPlate reflect sole-C-source metabolic competence and the activities of Gram-negative (G^-), Gram-positive (G^+) bacteria in and overall activity of soil microbial community. As shown in Fig. 1, during the whole incubation for 168 h, *AWCDs* for 99BC-4, 99BC-8 and SM3 had a steady rise in all 3 plates with incubation time, but exhibited differential sigmoidals of C substrate use, and the soils showed lower *AWCDs* in GP2 plate than in GN2 plate and EcoPlate, revealing that the G^+ bacteria in the soils possessed a weaker C source metabolic ability. The two *Bt* cotton lines performed inconsistently for the *AWCDs* since in all 3 plates, *AWCDs* for 99BC-4 were always significantly lower than those for 99BC-8 and SM3, suggesting that the metabolic activity of either G^+ or G^- bacteria in 99BC-4 rhizospheric soil were significantly decreased compared with those in its non-*Bt* recipient, and furthermore, significant differences also emerged between *AWCDs* for 99BC-8 and SM3 in the partial process of incubation, i. e., at the point of 24th h from start in GN2 plate; from 24th h to 84th h in GP2 plate; and from 36th h to 72nd h in EcoPlate. By comparing Fig. 1-a with Fig. 1-b, within the incubation from 48th h to 84th h, no significant differences in GN2 plate between *AWCDs* for 99BC-8 and SM3 but significantly higher *AWCDs* for SM3 than for 99BC-8 in GP2 plate, indicating that there appeared lower ratio of G^+ to G^- bacteria for 99BC-8 than for SM3. It is well in accordance with the observation for *Bt* and non-*Bt* maize[13]. Based on these results, it is speculated that the species and composition of the root exudates (Bt endotoxin included[8]) can play some selective role, to a certain degree, for both G^+/G^- bacteria and other microbes, and their periodic growth as well in the rhizospheric soil microbial community, which is among the most important biological characteristics for the rhizosphere. In addition, a higher Bt endotoxin content for 99BC-8 (though the significance of difference not related)[8], maybe leads to more microbes occurring, it seems then not difficult to understand higher *AWCDs* for 99BC-8 compared with those for 99BC-4.

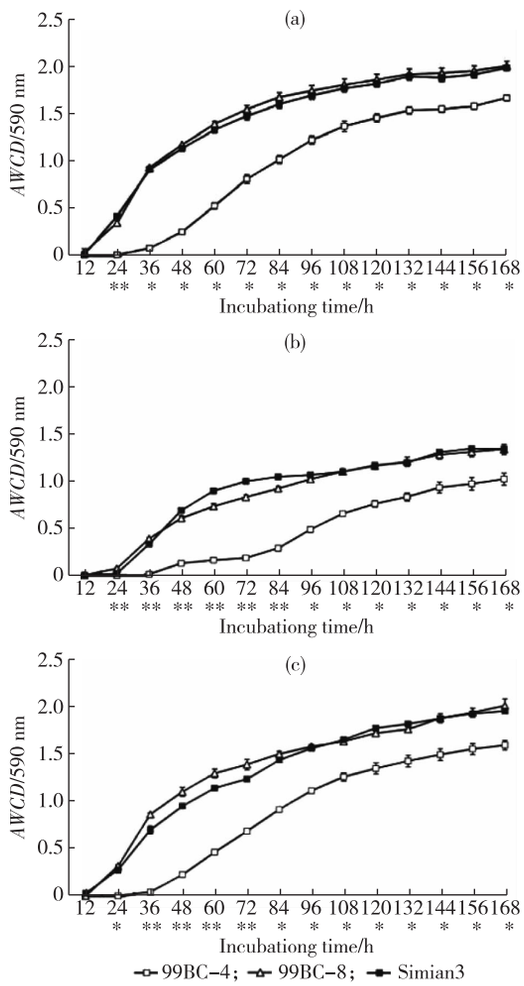

Fig. 1 AWCD kinetics for 99BC-4, 99BC-8 and SM3 on Biolog-GN2 (a), -GP2 (b) and -Eco (c) Plates. * indicates significant differences ($p < 0.05$) between AWCDs for 99BC-8/ SM3 and 99BC-4. ** indicates significant differences between any two of three ($p < 0.05$).

3.2 Functional diversity index variation of soil microbial communities

Functional diversity index between any two of three variations for 99BC-4, 99BC-8 and SM3 are presented in Table 1. It is clearly indicated that in GP2, GN2 plates and EcoPlate, *S*, *H'* and *U* for

99BC-4 had always remarkably lower values than those for 99BC-8 and SM3, which inferred that the catabolic diversity for 99BC-4 was reduced compared with that for SM3 whilst for 99BC-8, it was not the same case. 1/*D* and *ME* were able, to a certain degree, to distinguish the difference among the utilization of C sources by different rhizospheric microbial communities. 1/*D* differed in GP2 plate in the same way as *H'*, and had significant difference between 99BC-8 and 99BC-4 in GN2 plate, but no significant difference in EcoPlate. For *ME*, no significant difference was found in GP2 plate, significant differences observed the same way as for 1/*D* in GN2 plate, and significant differences the same way as for *U* in EcoPlate. In both GP2 and GN2 plates, *SE* for 3 microbial communities reflected the opposite differences to *H'*, but either no significant difference in EcoPlate. In GP2, GN2 plates and EcoPlate, *G* for 99BC-4 was significantly reduced compared with those for 99BC-8 and SM3, and besides, in GP2 plate, *G* for 99BC-8 also significantly reduced in comparison with that for SM3.

Table 1　Diversity and evenness indices for 99BC-4, 99BC-8 and SM3

		S	*G*	*H'*	*SE*	1/*D*	*U*	*ME*
GP	99BC-4	18.500 ± 0.289^{b}	0.170 ± 0.002^{b}	3.472 ± 0.029^{b}	0.099 ± 0.000^{a}	20.497 ± 1.518^{b}	6.196 ± 1.039^{b}	0.972 ± 0.005
	99BC-8	45.667 ± 2.728^{a}	0.361 ± 0.065^{b}	3.773 ± 0.126^{a}	0.069 ± 0.010^{b}	71.352 ± 16.086^{a}	12.837 ± 2.354^{a}	0.983 ± 0.004
	Simian3	50.667 ± 1.764^{a}	0.41 ± 0.021^{a}	3.934 ± 0.052^{a}	0.065 ± 0.002^{b}	77.607 ± 4.704^{a}	12.672 ± 0.328^{a}	0.983 ± 0.001
GN	99BC-4	53.000 ± 2.887^{b}	0.477 ± 0.025^{b}	4.020 ± 0.007^{b}	0.060 ± 0.001^{a}	105.968 ± 3.671^{b}	9.806 ± 1.484^{b}	0.988 ± 0.000^{b}
	99BC-8	78.667 ± 2.333^{a}	0.646 ± 0.072^{a}	4.297 ± 0.104^{a}	0.053 ± 0.003^{b}	125.73 ± 10.044^{a}	17.938 ± 2.683^{a}	0.991 ± 0.001^{a}
	Simian3	74.333 ± 1.667^{a}	0.612 ± 0.072^{a}	4.262 ± 0.000^{a}	0.054 ± 0.000^{b}	115.809 ± 1.899^{ab}	17.693 ± 1.0291^{a}	0.990 ± 0.001^{ab}
ECO	99BC-4	16.333 ± 0.471^{b}	0.37 ± 0.014^{b}	2.738 ± 0.006^{b}	0.869 ± 0.028	0.031 ± 0.000	5.892 ± 0.161^{b}	0.911 ± 0.007^{b}
	99BC-8	22.667 ± 1.247^{a}	0.576 ± 0.072^{a}	3.066 ± 0.124^{a}	0.899 ± 0.004	0.028 ± 0.002	9.679 ± 1.235^{a}	0.947 ± 0.010^{a}
	Simian3	23.333 ± 0.943^{a}	0.543 ± 0.023^{a}	3.044 ± 0.028^{a}	0.914 ± 0.002	0.029 ± 0.001	9.134 ± 0.263^{a}	0.948 ± 0.003^{a}

For each of GN2, GP2 and EcoPlates, different letters in apex in the same column indicate significant differences ($p < 0.05$).

3.3　Principal components analysis of metabolic function of soil microbial communities

PCA of each data set for 99BC-4, 99BC-8 and SM3 resulted in a PC score plot in which soil groups were separately located (Fig. 2,3). In each of the following 2 PC score plots (that in EcoPlate not shown), it was observed that on one hand, the soils were significantly divided into 2 groups along the positive direction of PC1 axis. PC1 scores differed significantly between 99BC-8, SM3 soil groups and 99BC-4 soil, and 99BC-4 soil had always a higher score for PC1 and showed a greater response to C sources in GP2, GN2 MicroPlates that correlated positively to PC1, compared with lower PC1 scores for the other soils. On the other hand, the soils were also significantly divided into 2 groups along the positive direction of PC2 axis. 99BC-8 soil located at the positive side, was greatly distinguished from 99BC-4, SM3 soil groups at the negative side, and showed a greater response to C sources in both MicroPlates that correlated positively to PC2, compared to lower PC2 scores for the other soils. The first 2 PCs accounted respectively for 35.59%, 19.37% in GN2 plate, and 30.57%, 18.89% in GP2 plate of total variation. Variation explained by 2 pairs of the first and second PC axes reached 54.96%, 49.46% respectively, indicating a better discrimination power of the microbial communities and metabolically functional diversity from different lines.

In other words, the PCA patterns shown in the *AWCD* data sets were strongly related to the use of individual C sources in GN2, GP2 plates. In GN2 plate, separation of the soil samples into 2 groups along PC1 was significantly affected by carbohydrates, carboxylic acids and amino acids. Substrates affecting the PC2 were carbohydrates and carboxylic acids. Some C sources, such as β-Methyl-D-glucoside (−0.971), Maltose(−0.915), Sucrose (0.939), D-Gluconic acid (0.969), L-Alanyl-glycine(−0.970), L-Threonine(0.932), Phenyethyl-amine(−0.953), Bromosuccinic acid (0.944) and α-D-Glucose-1-phosphate

(−0.937) had greater influence than the other substrates on the axis PC1. Thus, these C sources were primarily responsible for the separation along the PC1 axis. Similarly, Gentloblose (−0.813), Xylitol (0.861), β-Hydroxybutyric acid (0.957), L-Proline (−0.778), L-Alanine (−0.775) and α-Cyclodextrin (0.846) had the greatest influence on the PC2 axis and, thus, were important variables in the separation along the PC2 axis. In addition, in GP2 plate, separation along PC1 was significantly affected by carbohydrates and carboxylic acids. Substrates affecting the PC2 were carbohydrates. The G^+ bacteria for 99BC-4 were more likely to use such C sources as L-Alanyl-glycine (0.938), Xylitol (0.851), α-Methyl-D-glucoside (0.828), β-Hydroxybutyric acid (0.824) and Mannan (0.763) while the G^+ bacteria for 99BC-8, SM3 more likely to utilize some C sources such as p-Hydroxy- phenylacetic acid (−0.980), D-Galactose (−0.932), D-Mannose (−0.912) and D-Glucose-6-phosphate (−0.764). Similarly, the G^+ bacteria for 99BC-8 were more likely to metabolize such C sources as Turanose (0.885) and L-Pyroglutamic acid (0.883) whilst the G^+ bacteria for 99BC-4, SM3 more to catalyze the following C sources as Palatinose (−0.898) and α-Ketoglutaric acid (−0.784). It could be then, drawn that the C sources like β-Hydroxybutyric acid and Xylitol were more likely utilized by both the G^+ bacteria for 99BC-4 and the G^- bacteria for 99BC-8.

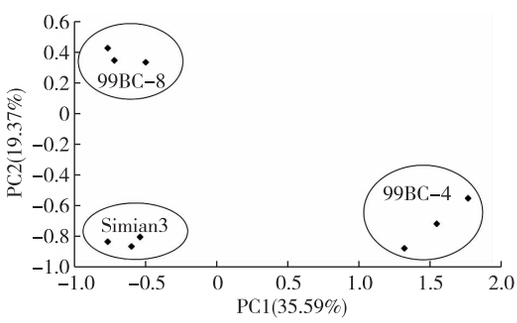

Fig. 2 Principal components analysis of carbon utilization for 99BC-4, 99BC-8 and SM3 in GN2 plate

4 Discussion

Bt cottons can produce crystal toxic protein for protecting themselves against the insects *Helicoverpa armigera*, even suppressing these bollworm in multiple crops in area with *Bt* cotton[2], which seems to justify the large-scale release of these transgenic cotton varieties. Nevertheless, the ecological and environmental risks of them, such as the impacts on non-target organisms, are widely concerned with[7].

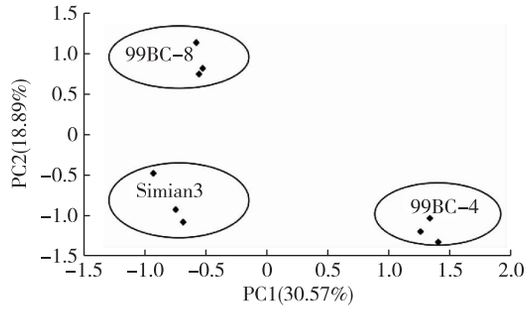

Fig. 3 Principal components analysis of carbon utilization for 99BC-4, 99BC-8 and SM3 in GP2 plate

Since Bt protein can be released into soil from different crop sectors [14-16], and wastes from the animals living on *Bt* cotton vegetative parts[17], the potential risks of *Bt* cotton on the soil microbial communities should be paid more attention.

There exist some interactions between crop root exudates and rhizospheric soil microbial communities. Some rhizospheric microbes can affect root exudates through changing the content level of the allelopathic compounds, and vice versa, the allelopathic compounds can also affect non-target soil rhizospheric microbial communities. Although microbial biomass indicated microbial community size, Biolog was a method of analyzing the potential metabolic diversity of soil microbial community. Biolog has been widely used in assessing microbial metabolic diversity in agricultural soils. Over the years, increased understanding of the Biolog assay has demonstrated the reproducibility of Biolog profiles and supported the theory that shifts in Biolog metabolic diversity patterns are related to shifts in community composition.

The *AWCD* reflects the sole-C-source utilization ability of the soil microbial community and soil microbial activity[9]. *H'* is a measure of actual richness and evenness of the microbial community, *U* is characterized of the diversity of C source utilization competence of mi-

crobial community, *S* is a reflection of the total number of C substrates utilized generally and defined as the number of different groups of microbes found occurring, whilst *SE* reflects the comparability of substrate utilization between all utilized substrates, and the expected distribution of microbial groups within the community[18-19]. 1/*D* is an indicator of dominant types of microbes whereas *G* emphasizes the differences of the microbial community between in utilizing single C substrate. According to our results, it can be deduced that the rhizospheric soil samples of 99BC-8 and SM3 showed a larger richness and diversity of, and centered more dominant species in the microbial communities, with a lower comparability of metabolized substrates, thus reflecting a high metabolic potential and functional diversity in soil microbial communities, though there emerged differences between them, involving a greater quantity of microbes without activity within 99BC-8 rhizospheric soil sample. But more likely it is the releasing of Bt endotoxin into rhizospheric soil from transgenic *Bt* cotton that selectively or in varying degrees, affected both G^+/G^- bacteria and other microbes, and their growth at different stages in the rhizospheric soil. In other words, the presence of Bt endotoxin likely stimulated the growth of some specific microbes by using their preferential C sources, which seemed to dominate the microbial responses in the soil although inconsistent responses in the two transgenic *Bt* lines. For instance, β-Hydroxybutyric acid and Xylitol were more likely utilized by the G^+ bacteria for 99BC-4 and the G^- bacteria for 99BC-8. As for the microbial communities and metabolically functional diversity, the soil samples were discriminated from one another rationally.

Correlations between soil microbial functional diversity and organic C have been reported[20]. The importance of organic C in influencing the variation of soil microbial functional diversity was also concerned[21]. Another report held that there was a significantly positive correlation between the total soil microbial number, *AWCD*, C_{mic}, and *H'* [22]. However, rather than supporting these assumptions, from this study, the positive correlation is not involved with C_{mic}. Of course, further investigations (for example, using different soil types and microbial diversity analysis techniques) of the relationship would be necessary to substantiate this conclusion.

Moreover, the data obtained showed that there was, to some extent, a positive correlation between Fe content[8], *S*, *AWCD*, and *H'*, *U* although redundancy analysis (RDA) was not used to visualize the relationships between microbial parameters and soil chemical properties.

Biolog technique has widely been used in part for its simplicity, availability of automated measuring apparatus, and yield of a great deal of information about an important functional attribute of microbial communities. Frankly speaking, however, the fundamental basis of using such an approach for ecological studies has been questioned on account of its dependence on inefficient extraction, the physiological status of inoculated cells, the subsequent growth of cells, the relevance and concentrations of the C substrates used[23], and still, the complicated data analysis[24], the degree to which the results reflect function rather than structure[25]. Nevertheless, such methods have been shown in some cases to be as sensitive as or more sensitive than measuring microbial biomass and respiration[26], and they have been used to detect the effects of important environmental changes on sensitive ecosystems[27], even if the effects have likely come from the changes in the physio-biochemical properties of transgenic *Bt* cottons for genetic amendments, rather than from the direct action of the expression product, Bt protein[28].

5 Conclusions

The widely planting of transgenic crops has made a research hit of the risks potentially imposed to soil microbes and agro-ecosystem. Combined with the earlier study[8], it is concluded that in the rhizospheric soils which had been planted by transgenic *Bt* cotton lines (99BC-4, 99BC-8), Cry1Ac Bt toxic protein changed soil nutrient conditions such as increasing N, Ca, Zn, Co and Cu but decreasing K and Mg contents. Bt endotoxin triggered more non-target soil microbes appearing in the rhizosphere, significantly raising C_{mic}, to degrade more rapidly such a cotton-re-

leased allelopathic compound, but inhibited severely the growth of the microbes at work in 99BC-8 rhizospheric soil. Bt endotoxin affected selectively microbial composition, altered the utilization patterns of C sources, promoted the microbial functional diversity, and consequently, evolved into a better discrimination among the transgenic *Bt* lines and their non – *Bt* recipient from one another.

To sum up, transgenic *Bt* cottons pose effects on soil ecosystem, especially on rhizospheric soil nutrition and microbial communities. And the possibility of aftermath effects from transgenic crops cannot be excluded and environmental impact assessments must be examined on a case-by-case basis. We are, hereby, more inclined to hold that the research on the safety of transgenic crops is also a long-term and arduous task, which relies on systematic accumulative work for dozens of years.

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5 生根与移栽

君子兰组织培养研究表明,生根有2种方式,一种是直接在分化培养基上生根,另一种是将无根芽苗转移到专门的生根培养基上培养生根[4-5,10]。具体的生根培养基配方见表1。当试管苗生根培养长出小叶3~4片后,就可进行试管苗的移栽了。移栽时注意经过7~14 d的炼苗后再进行正常的栽培管理[16]。

6 问题与展望

君子兰是多年生常绿草本植物,可观可赏,全株入药。除美化环境外,还有一定的药用价值[18]。君子兰植株体内含有石蒜碱和君子兰碱,还含有微量元素硒,药物工作者利用含有这些化学成分的君子兰株体进行科学研究,用来治疗癌症、肝炎病、肝硬化腹水和脊髓灰质病毒等。通过试验证明,君子兰叶片和根系中提取的石蒜碱,不但有抗病毒作用,而且还有抗癌作用[18]。植物次生代谢与调控正在受到越来越多专家的关注,君子兰株体中含有的抗病毒及抗癌的物质有些就属于次生代谢物,而这些代谢物很难在体外合成,因此对君子兰次生代谢的研究是今后组织培养的重要方向。

虽然人们在君子兰组织培养方面取得了一定进展,也成功建立过植株再生体系,但其研究结果存在很大差异,难以建立一套完整、高效、通用的植株再生体系[16]。另外,君子兰体细胞胚胎发生途径的研究还未见报道,因此,需要加强研究,找到不同品种、不同外植体最适的培养基配方;需要在组织培养过程中进行培养基筛选、机理研究,来建立一套完善的植株再生体系,为君子兰优良植株的扩繁提供依据。

鉴于现有的研究成果还未在生产实践中转化,工厂化育苗的开展更无从谈起。组织培养苗都是在培养环境最佳情况下获得的,需要经过炼苗后才能适应外界的环境。而关于炼苗移栽方面的研究也很少,这也是今后君子兰组织培养研究的方向。

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