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*Short Communication*

# A Comparative Study of CN-Resistant Respiration in Different Cultures of Tobacco Callus[1]

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## ABSTRACT

**The callus of *Nicotiana rustica* cv Gansu yellow flower and *N. tabacum* cv willow leaf were cultured on ordinary subculture medium (M-1) and on regeneration medium (M-2), respectively. No differentiation was observed in Gansu yellow flower tobacco callus cultures grown on both M-1 and M-2 medium. The respiration of both cultures was partially resistant to cyanide and markedly inhibited by *m*-chlorobenzhydroxamic acid. The relative contributions of alternative and cytochrome pathway were 31% and 47% of the total respiration, respectively, in M-1 callus cultures. The relative $O_2$ uptake of the two pathways was not changed significantly in M-2 callus cultures. In subcultured M-1 callus cultures of Willow leaf tobacco, the respiration mediated via alternative pathway was about 29 to 38% of the total respiration, and the cytochrome pathway still was the major respiratory pathway. In M-2 callus cultures in which differentiation occurred, the relative contribution of alternative pathway increased to 41 to 47% of the total respiration, and the cytochrome pathway decreased considerably. These results suggested that the change of respiratory electron transport pathway was probably related to the differentiation of tobacco callus cultures.**

The wide occurrence of CN-resistant respiration in higher plants has received increased attention in recent years. It has been found that CN-resistant respiration is associated with flowering in some plants (4, 6), seed germination (17), the climacteric rise of fruit respiration (9, 14) and wound-induced respiration of bulky storage organs (5, 10, 11). Some hypotheses have been proposed to explain the mechanism of the development of CN-resistant respiration (2, 7). However, there is no report concerning the relation of CN-resistant respiration to tissue differentiation. We selected the callus of two varieties of tobacco with different differentiation potential as experimental materials. One is the callus of *Nicotiana rustica* cv Gansu yellow flower which loses its differentiation potential when subcultured for some time, and the other is the callus of *Nicotiana tabacum* cv Willow leaf which possesses a greater ability to differentiate. By comparative study of CN-resistant respiration of tobacco callus cultures grown on the two media, we attempted to determine the extent of participation of the CN-resistant respiration in tobacco callus under differentiation and nondifferentiation conditions. The object of this study is to elucidate the relationship of respiratory electron transport pathway to tissue differentiation of tobacco callus.

## MATERIALS AND METHODS

Two tobacco calli derived from unpollinated young ovaries were transplanted on subculture medium (M-1) and on regeneration medium (M-2). The minerals and organic compounds of MS medium (12) were used as the basic components in both cases. The M-1 medium was supplemented with 6-BA (0.5 mg/l) and 2,4-D (2 mg/l), but in M-2 medium 6-BA (2 mg/l) and IAA (1 mg/l) were added. The callus cultures were placed in a growth cabinet maintained at 28°C, and illuminated with fluorescent light (3,000 lux) for 12 h/d. Observations were made on the 10th, 15th, 20th (or 21st), and 25th d of culture. The callus cultures grown on M-1 medium and M-2 medium are called M-1 callus and M-2 callus, respectively.

Cytological observations were made by crush method and staining with acetic acid-lichen red. The $O_2$ uptake of the culture tissue suspension (0.15 g/2 ml) was measured polarographically using a membrane oxygen electrode (8). The respiratory rate of tobacco callus cultures was measured in phosphate buffer solution (pH 6.8, 0.15 M). The respiratory rate was indicated by consumption of $\mu$l $O_2$/g fresh weight·h.

The existence of CN-resistant respiration was determined using KCN and *m*-CLAM[2] as selective inhibitors of the respiratory pathway (13). A given volume of solutions of either KCN (pH 6.8) or *m*-CLAM (pH 6.8) was injected separately and in combination into the stirred cell-containing culture suspension to give a final inhibitor concentration of 1 mM in the medium.

The extent of actual operation of the alternative pathway and the Cyt pathway as well as their relative contributions to the total respiration were determined by Bahr and Bonner's hydroxamic acid titration method (1). The data were calculated with the following equation as modified by Theologis and Laties (16):

$$V_T = \rho \cdot V_{alt} + V_{cyt} + V_{res}$$

where $V_T$ is the total respiratory rate, $V_{cyt}$ is the Cyt-mediated respiration, $V_{res}$ is the residual $O_2$ uptake which is not inhibited by CN plus *m*-CLAM, $\rho$ represents the fraction of the alternative pathway which is operating, and $V_{alt}$ is the maximal capacity of the alternative pathway. Accordingly, $\rho \cdot V_{alt}$ represents the extent of actual operation of the alternative pathway in the absence of inhibitor. The ratio of $\rho \cdot V_{alt}/V_{cyt}$ represents the degree of relative contribution of the two pathways.

**Chemicals.** *m*-CLAM was synthesized by Professor Y. L. Li (Institute of Organic Chemistry, Lanzhou University). 6-BA was obtained from Shanghai Institute of Biochemistry, Academia Sinica. IAA and other chemicals were products of Shanghai Chemical Reagent Plant.

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[2] Abbreviation: *m*-CLAM, *m*-chlorobenzhydroxamic acid.

Table I. *Contributions of Various Respiratory Components in M-1 and M-2 Callus of Gansu Yellow Flower Tobacco*

| Calli | Time in Culture | $V_T$ | $V_{alt}$ | $\rho$ | $\rho \cdot V_{alt}$ | $V_{cyt}$ | $V_{res}$ | $\frac{\rho \cdot V_{alt}}{V_{cyt}}$ |
|---|---|---|---|---|---|---|---|---|
| | *d* | *μl $O_2$/g fresh wt·h* | | | *μl $O_2$/g fresh wt·h* | | | |
| M-1 callus | 10 | 186.2 (100)* | 80.9 | 0.71 | 57.4 (30.8) | 82.0 (44.0) | 46.8 (25.1) | 0.70 |
| | 15 | 175.6 (100) | 81.9 | 0.75 | 61.4 (34.9) | 79.0 (45.0) | 35.1 (19.9) | 0.77 |
| | 21 | 196.7 (100) | 88.5 | 0.73 | 64.6 (32.8) | 80.9 (41.1) | 49.1 (25.0) | 0.79 |
| | 25 | 193.3 (100) | 82.3 | 0.65 | 53.5 (27.6) | 104.0 (53.8) | 35.8 (18.5) | 0.51 |
| M-2 callus | 10 | 244.8 (100) | 117.6 | 0.68 | 79.9 (32.6) | 112.1 (45.8) | 52.6 (21.5) | 0.71 |
| | 15 | 266.1 (100) | 122.4 | 0.67 | 82.0 (30.8) | 125.5 (47.1) | 58.5 (21.9) | 0.65 |
| | 21 | 216.4 (100) | 103.8 | 0.71 | 73.7 (34.0) | 98.9 (45.7) | 43.7 (20.1) | 0.74 |
| | 25 | 204.0 (100) | 81.1 | 0.63 | 51.1 (25.0) | 105.2 (51.5) | 47.7 (23.3) | 0.49 |

[a] Data in parentheses are the relative contributions of various respiratory components (as % of $V_T$).

Table II. *Respiratory Rate of M-1 and M-2 Callus in Willow Leaf Tobacco with added KCN and m-CLAM*

| Calli | Time in Culture | Respiratory Rate | | | |
|---|---|---|---|---|---|
| | | Control ($H_2O$) | KCN (1 mM) | m-CLAM (1 mM) | KCN + *m*-CLAM |
| | *d* | *μl $O_2$/g fresh wt·h* | | | |
| M-1 callus | 10 | 90.8 (100)[a] | 46.8 (51.5) | 39.2 (43.2) | 16.8 (18.5) |
| | 15 | 90.0 (100) | 45.4 (50.4) | 44.8 (49.7) | 12.6 (14.0) |
| | 20 | 86.7 (100) | 42.7 (49.2) | 31.3 (36.1) | 11.8 (13.6) |
| | 25 | 91.5 (100) | 50.7 (55.4) | 31.1 (33.9) | 15.8 (17.3) |
| M-2 callus | 10 | 79.8 (100) | 56.0 (70.1) | 39.6 (49.6) | 18.6 (23.3) |
| | 15 | 78.6 (100) | 57.2 (72.7) | 35.7 (45.4) | 21.7 (27.6) |
| | 20 | 78.1 (100) | 55.5 (71.1) | 32.7 (41.9) | 17.3 (22.2) |
| | 25 | 83.0 (100) | 61.0 (73.5) | 36.8 (44.4) | 19.0 (22.8) |

[a] Data in parentheses are the % of control.

## RESULTS AND DISCUSSION

**CN-Resistant Respiration in Callus of *N. rustica* cv Gansu Yellow Flower.** Microscopic observations showed that, in M-1 callus cultures, many meristematic cells were formed during the culture period, but no tissue differentiation and organ formation were observed. In M-2 callus cultures, more meristematic cells were formed and the cell multiplication was faster than that of M-1 callus. However, there was no apparent differentiation of tissue and organ throughout the period of culture, except a few tracheids were formed in a few cultures. The enlargement of the callus volume was faster than that of M-1 callus.

According to the inhibition experiments, the respiration of M-1 callus was not only relatively resistant to CN, but also markedly inhibited by *m*-CLAM. The CN-insensitive respiration was 61 to 69% of the control, and *m*-CLAM inhibited the total respiration by 28 to 34%. The addition of CN and *m*-CLAM in combination inhibited the respiration by 75 to 82%. As compared with M-1 callus, the respiratory rate of M-2 callus was higher, but the capacity of the CN resistance and the percentage of inhibition by *m*-CLAM were essentially similar in Gansu yellow flower tobacco callus cultures grown on both media.

Using the hydroxamic acid titration method, the extents of actual operation of the two electron transport pathways and their relative contributions to the total respiration of the callus cultures were measured (Table I). In M-1 callus, $\rho \cdot V_{alt}$ generally maintained an oxygen uptake rate of 57 to 64 μl $O_2$/g fresh weight·h, which amounted to 30 to 34% of $V_T$. The value of $V_{cyt}$ was higher, reached a $Q_{O_2}$ of 79 to 82, and contributed 41 to 45% to the total respiration. The average ratio of $\rho \cdot V_{alt}/V_{cyt}$ was 0.75, and fell to 0.51 at the 25th d after transplant. In nondifferentiation M-2 callus cultures, average $\rho \cdot V_{alt}$ was 73 to 82 μl $O_2$/g fresh weight·h before 21 d in culture, and the relative contribution of it to $V_T$ was 30 to 34%, which decreased slightly by the 25th d in culture. $V_{cyt}$ remained relatively constant over the whole period, the relative contribution to $V_T$ being 45 to 51%. The average ratio of $\rho \cdot V_{alt}/V_{cyt}$ was 0.7 during the culture of 21 d, which fell to 0.49 at the 25th d as a result of a decrease in the relative contribution of the alternative pathway.

The experimental results indicate that in callus cultures of Gansu yellow flower tobacco, although the operation of the CN-resistant, alternative pathway was considerable, the cytochrome pathway was still the major pathway. The addition of different hormones to the medium did not produce a significant change

Table III. *Contributions of Various Respiratory Components in M-1 and M-2 Callus of Willow Leaf Tobacco*

| Calli | Time in Culture | $V_T$ | $V_{alt}$ | $\rho$ | $\rho \cdot V_{alt}$ | $V_{cyt}$ | $V_{res}$ | $\frac{\rho \cdot V_{alt}}{V_{cyt}}$ |
|---|---|---|---|---|---|---|---|---|
| | *d* | *μl $O_2$/g fresh wt·h* | | | *μl $O_2$/g fresh wt·h* | | | |
| M-1 callus | 10 | 90.8 (100)[a] | 30.0 | 0.9 | 27.0 (29.7) | 47.0 (51.7) | 16.8 (18.5) | 0.57 |
| | 15 | 90.0 (100) | 32.7 | 1.0 | 32.7 (36.4) | 44.6 (49.5) | 12.6 (14.0) | 0.73 |
| | 20 | 86.7 (100) | 30.8 | 1.0 | 30.8 (35.5) | 44.0 (50.7) | 11.8 (13.6) | 0.70 |
| | 25 | 91.5 (100) | 34.9 | 1.0 | 34.9 (38.1) | 40.8 (44.5) | 15.8 (17.3) | 0.85 |
| M-2 callus | 10 | 79.8 (100) | 37.3 | 1.0 | 37.3 (46.7) | 23.8 (29.8) | 18.6 (23.3) | 1.57 |
| | 15 | 78.6 (100) | 35.4 | 0.92 | 32.6 (41.5) | 24.2 (30.8) | 21.7 (27.6) | 1.35 |
| | 20 | 78.1 (100) | 38.1 | 0.92 | 35.1 (44.9) | 25.6 (32.8) | 17.3 (22.2) | 1.37 |
| | 25 | 83.0 (100) | 42.0 | 0.93 | 39.1 (47.1) | 24.8 (29.9) | 19.0 (22.8) | 1.57 |

[a] Data in parentheses are the relative contributions of various respiratory components (as % of $V_T$).

in the relative contribution of the two electron transport pathways in tobacco callus cultures under nondifferentiation conditions.

**Change of CN-Resistant Respiration in Callus Cultures of *N. tabacum* cv Willow Leaf under Differentiation Condition.** Microscopic observations showed that, in contrast to the M-1 callus cultures, in which no apparent tissue differentiation took place throughout the period of culture, the M-2 callus cultures grown on regeneration medium differentiated into tracheids at the 10th d and vascular systems by days 15 to 20. Embryoids and bud primordia had been produced by the 25th d. Green bud nodules could be seen by the naked eye after 45 d in culture.

The inhibitory effects of CN and *m*-CLAM on respiration of callus cultures grown on two different media exhibited a marked difference as shown in Table II. In M-1 callus cultures, the CN-resistant respiration was 49 to 55% of the total respiration, and *m*-CLAM inhibited the respiration by 51 to 66% during the culture period of 25 d. When CN and *m*-CLAM were added in combination, 82 to 87% of the total respiration was inhibited. In M-2 callus cultures, the greater part of respiration which amounted to 70 to 73% of total respiration was resistant to CN throughout the period of culture. One mM *m*-CLAM alone inhibited the respiration by 51 to 59%, and the inhibition increased to 73 to 78% in the presence of CN. In contrast to M-1 callus, the M-2 callus cultures exhibited a higher resistance to CN during the period of tissue differentiation. The extents of actual operation of the alternative and Cyt pathways can not be calculated from the data obtained by inhibition experiments (in Table II). There is a diversion of electron flux between the two electron transport pathways in the presence of inhibitor (2), so that the sum of $O_2$ uptake inhibited by CN and by *m*-CLAM is greater than the total respiration.

Using the hydroxamic acid titration method, the extents of actual operation of the two electron transport pathways and their relative contributions to the total respiration were calculated in Willow leaf tobacco callus cultures grown on both media (Table III). In M-1 callus cultures, $\rho \cdot V_{alt}$ amounted to 27 to 34 $\mu$l $O_2$/g fresh weight·h and was 29 to 38% of $V_T$. The $Q_{O_2}$ of $V_{cyt}$ was 40 to 47 and was 44 to 51% of $V_T$. Later, it exhibited a tendency to fall. The average ratio of $p \cdot V_{alt}/V_{cyt}$ was 0.71. The $V_{res}$ was below 20% of $V_T$. These results showed that in the respiration of M-1 callus cultures, about one-third of the respiratory electron flux was transported through an alternative pathway, whereas the bulk of respiratory electron flux was mediated by the Cyt pathway. The relative contributions and the ratios of the two electron transport pathways were approximately the same as those of nondifferentiated Gansu yellow flower tobacco callus cultures. Compared with M-1 callus cultures, the participation of the different electron transport pathways exhibited a marked difference in M-2 callus cultures of Willow leaf tobacco. The relative contribution of alternative pathway increased by up to 41 to 47% of $V_T$ (data in parentheses), although the value of $\rho \cdot V_{alt}$ was essentially unchanged. The $V_{cyt}$ value decreased considerably, and was only 29 to 32% of $V_T$. The average ratio of $\rho \cdot V_{alt}/V_{cyt}$ which represents the degree of relative contribution of the two pathways was 1.46. Another difference in M-2 callus cultures was the rise in $V_{res}$, which amounted to 22 to 27% of $V_T$. These results showed that during the period of tissue differentiation of Willow leaf tobacco callus cultures, the participation of alternative pathway was higher than that of their Cyt pathway, and that the major part of the respiratory electron flux was transported through the alternative pathway. Based on the results with Gansu yellow flower tobacco callus in which the inhibition of respiration by CN and *m*-CLAM was essentially similar in both media, the influence of exogenous hormones on CN-resistant respiration might be excluded; thus, the correlation between tissue differentiation and change of the respiratory electron transport pathways in Willow leaf tobacco callus cultures became evident.

There is currently no information concerning the relationship between callus differentiation and CN-resistant respiration, but from the results of Brown and Thorpe (3) indirect evidence may be obtained. They found that the tobacco callus cultures had higher levels of adenosine phosphate and $NAD^+$, as well as lower levels of energy charge and NADH during morphogenesis of meristemoids and initiation of shoots. It is generally considered that the alternative pathway is a nonphosphorylative electron transport pathway (15). Thus, it is assumed that during the period of differentiation in tobacco callus, the rise in the alternative pathway and the drop in the Cyt pathway will cause a decrease in energy charge; and as the electron flux is not controlled by phosphorylation, the decline of NADH and the rise of $NAD^+$ can be expected. These assumptions agree with the results of Brown and Thorpe (3).

Although the correlation between tissue differentiation and change of respiratory electron transport pathway in tobacco callus cultures is obvious, the question whether the change of respiratory pathway controls callus differentiation or callus differentiation causes the change in respiratory pathway remains to be solved. It is also not known whether the correlation between tissue differentiation and respiratory pathway also exists in other plant materials. These questions remain to be investigated in the future.

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