

## Biological Characteristics of Benzimidazole-Resistant and -Sensitive Isolates of *Monilinia fructicola* from Peach Fruits in Korea

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Fungicide-resistant isolates of *Monilinia fructicola* grew readily on media amended with 0.1, 1.0, 10, 100 and 1,000 µg a.i./ml of carbendazim, benomyl, or thiophanate-methyl. However, sensitive isolates did not grow on media amended even with 0.1 µg a.i./ml of carbendazim, 1.0 µg a.i./ml of benomyl or thiophanate-methyl. The fitness compositions including mycelial growth on fungicide-free medium, sporulation on fungicide-free medium and pear, and virulence on pear were not different between resistant and sensitive isolates. The resistant isolates persisted carbendazim resistance during multiple subculturing and long term storage. The competitive ability of resistant isolates obtained from peach orchards in Korea was similar to those of sensitive isolates.

**Keywords :** competitive ability, fitness compositions, fungicide resistant, *Monilinia fructicola*.

The brown rot of stone fruits caused by *Monilinia fructicola* is one of the most destructive diseases in their production process in Korea. *M. fructicola* usually causes blossom blight in spring, and the disease provides inoculum for latent infection of green fruits. Brown rot appears on fruit around the end of the season or during storage, transport, and even at the markets (Agrios, 1997).

The control of the brown rot depends on chemical method using fungicides (Agrios, 1997; Delp, 1988). The advent of many effective fungicides has resulted in a reasonable control in recent years. However, in the past two decades, fungal resistance to fungicides has become an increasingly important problem (Delp, 1988; Johnes and Ehert, 1976; Keinath and Zitter, 1994; Tate et al., 1974; Zehr et al., 1991). All around the world, many plant pathogens, for example, *Botrytis cinerea* (Delp, 1998; Kim et al., 1995), *Penicillium digitatum* (Bus et al., 1991), *P. italicum* (Bus et al., 1991), *Venturia inaequalis* (Koernaat et al., 1992), *Uncinula necator* (Pearson and Taschenberg, 1980),

*Coccomyces hiemalis* (Johnes and Ehert, 1980), and *Didymella bryoniae* (Keinath and Zitter, 1994), have been reported for their resistance to benzimidazole fungicides. Benomyl resistance of *M. fructicola* was initially reported by several authors from late 1970s (Johnes and Ehert, 1976; Szkolink and Gilpatrick, 1977). Benomyl resistant isolates have shown cross-resistance to another benzimidazole fungicides (Kim et al., 1995; Lim et al., 1998). Sonoda and Ogawa (1982) reported that benomyl-sensitive isolates showed higher growth rate than benomyl-resistance isolates on PDA and peach fruits. In New Zealand, Sanoamuang and Gaunt (1985) reported that subpopulation of carbendazim-resistant isolates in *M. fructicola* populations maintained permanently and pathogenicity, virulence, and fitness of carbendazim-resistance isolates were similar to the original values after survival for 1 year.

Fungicide resistance of *M. fructicola* in Korea, benzimidazole-resistant isolates from overwintering mummies and peduncles in several locations were first reported by Lim et al. in 1998. Also, they reported that some benomyl-resistant isolates showed cross-resistance to other benzimidazole fungicides and double resistance to dicarboximide fungicides (Lim et al., 1998).

In this paper, we report studies on mycelial growth on agar medium, spore germination, virulence, and competitive ability on canned peach, sporulation on agar medium and pear, and the stability of benzimidazole-resistant isolates.

### Materials and Methods

**Fungicides.** The following fungicides were used; benomyl (50% WP), carbendazim (60% WP), and thiophanate-methyl (70% WP). Fungicide suspensions prepared were diluted to concentration, and added to potato dextrose agar (PDA) of which temperature is between 50-60°C, immediately before pouring in Petri plates (Zehr et al., 1991).

**Fungal isolates.** Benzimidazole-resistant (four isolates) and sensitive (four isolates) isolates obtained from brown rot peach fruits at the middle of the season in 1998 at Chungdo and Kyongsan, Korea.

**Mycelial growth.** Fungicides-resistant and sensitive isolates

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were tested for growth on fungicide-free PDA and on PDA amended with 0.1, 1.0, 10, 100, and 1000  $\mu\text{g}$  a.i./ml of benomyl, carbendazim, and thiophanate-methyl. The 5-mm-diameter mycelium plugs were taken from the margin of 7-day-old PDA culture and transferred to the test media. The colony diameters were examined after incubation for 7 days at 25°C.

**Sporulation, spore germination and virulence.** *In vitro*, sporulation of fungicides-resistant and sensitive isolates were tested on 15-day-old PDA culture in the dark. The conidial concentration was examined by a haemocytometer after harvest with 50 ml sterilized distilled water per plate. For the spore germination test, spore concentration was  $1 \times 10^5$  conidia/ml and the suspension was incubated for 8 hr at 25°C. Spore germination was checked twice for replication and counted from 300 spores in each trial.

Sporulation rates of fungicides-resistant and sensitive isolates were examined *in vivo* on fungicide-free pear and on pear treated with 1.0  $\mu\text{g}$  a.i./ml of carbendazim. Inoculum was taken from the margin of 7-day-old PDA culture. The inoculated pears were stored in a plastic tray which was wrapped with the plastic bag and kept at 25°C in the dark. Incubation period and latent period were investigated at intervals of 1 hr after 15 hr. Lesion sizes were measured 7 days later (Elmer and Gaunt, 1994).

**EC<sub>50</sub> values in subculture conditions.** For four months, all the isolates were subcultured once a week. In the 5<sup>th</sup> subculture, responses to fungicide were tested on PDA free of fungicide or incorporated with 0.1, 1.0, 10, 100, 1000  $\mu\text{g}$  a.i./ml of carbendazim. EC<sub>50</sub> values of all the isolates were compared with the original values. After storage for four months on slant PDA, the responses were checked with the similar method to subculture condition.

**Stability in storage.** After 7 days of incubation on fungicide-free PDA, 5-mm-diameter mycelial plugs were taken from the margin of colony and transferred to fungicide-free PDA slant. Inoculated PDA slants were incubated at 25°C for 7 days and transferred to 4°C. After storage for 16 weeks, all isolates were subcultured on fungicide-free PDA for 7 days. Responses to fungicide were tested on PDA amended with 0.01, 0.1, 1.0, 10, 100, and 1000  $\mu\text{g}$  a.i./ml of carbendazim.

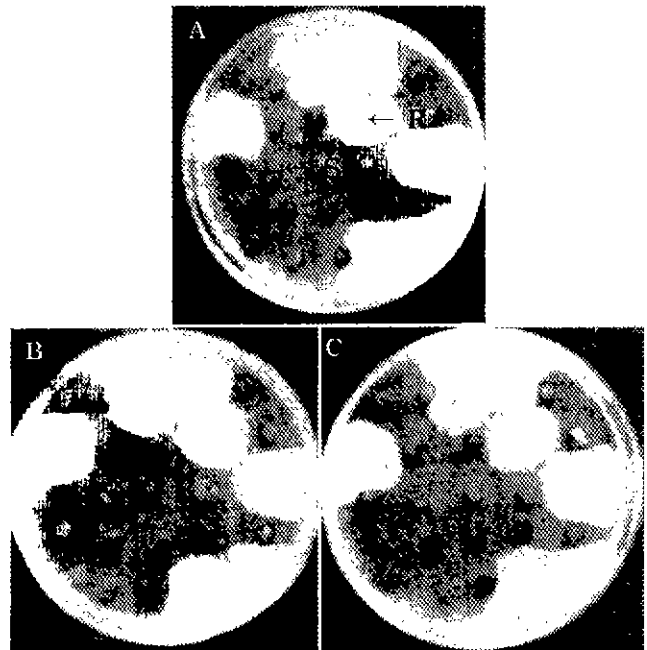
**Competitive ability.** One benzimidazole-resistant isolate, K726, and the other benzimidazole-sensitive, K765, were used in this study. The competitive behavior of the two isolates in the absence of fungicide was investigated on canned peach fruit. Conidial suspensions of the two isolates were adjusted to  $1 \times 10^5$  conidia/ml, and mixed to give a proportion of resistant isolate from 0% to 100% at 20-30% intervals. The suspension was inoculated on canned peach fruits by spray and the treated fruits were incubated on a plastic tray which was wrapped with the plastic bag at 25°C in the dark. After 7 days of incubation, conidia on each fruit were harvested in 50 ml sterilized distilled water. Responses to fungicide were tested on PDA amended with 10  $\mu\text{g}$  a.i./ml of carbendazim. Density of harvested conidia were adjusted to  $1 \times 10^5$  conidia/ml and 200 aliquots was pipetted onto PDA plates amended and unamended. Conidia in the aliquots were distributed evenly over the surface of the plates by a glass rod and germinated spores were counted after 8 hours of incubation. Germination rates of conidia on the amended medium indicated the ratio of

fungicide-resistant spores.

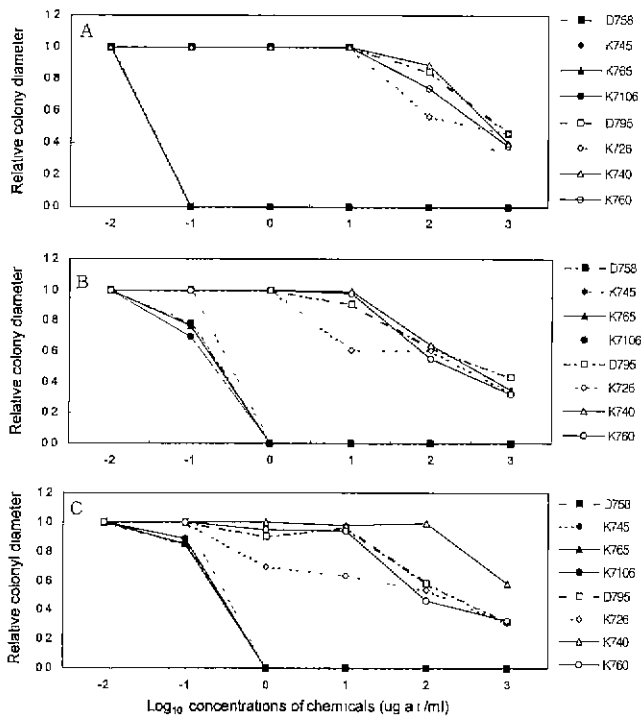
## Result

**Fungicide resistance test.** Of the eight isolates used in the first test, four isolates (D758, K745, K765, and K7106) were sensitive to carbendazim, benomyl and thiophanate-methyl (no growth at 1.0  $\mu\text{g}$  a.i./ml). On the other hand, the other four isolates (D795, K726, K740, and K760) showed fungicide resistance (growth at 1.0  $\mu\text{g}$  a.i./ml of carbendazim, benomyl, and thiophanate-methyl) (Fig. 1). On the medium containing 0.1  $\mu\text{g}$  a.i./ml of carbendazim, the rates colony diameter relative to fungicide free medium was 0 for the sensitive isolates, but those of resistant isolates were from 0.38 to 0.46 at 1,000  $\mu\text{g}$  a.i./ml (Fig. 2A). Carbendazim resistant isolates showed reduced sizes of colony diameter at 10  $\mu\text{g}$  a.i./ml (Fig. 2A). For benomyl and thiophanate-methyl (Fig. 2B, C), sensitive isolates showed no colony growth at 1.0  $\mu\text{g}$  a.i./ml. However, the isolates D795, K726, K740, and K760 which had longer than 0.5 of colony diameter rates on media amended with 100  $\mu\text{g}$  a.i./ml of benzimidazole fungicides, were considered highly resistant to carbendazim, benomyl, and thiophanate-methyl (Fig. 2).

**Mycelial growth.** The mycelial growth was not significantly different ( $P=0.05$ ) between carbendazim-resistant



**Fig. 1.** Responses of *M. fructicola* isolated from peach orchards in Korea to fungicides. Test media were amended with either 1.0  $\mu\text{g}$  a.i./ml of benomyl (A), carbendazim (B), or thiophanate-methyl (C). R and S represent resistant and sensitive isolate to the fungicides.



**Fig. 2.** Relative growth of benzimidazole-resistant (vacant symbols) and -sensitive (filled symbols) isolates on PDA amended with 0.01, 0.1, 1.0, 10, 100, and 1000 µg a.i./ml of a certain fungicide A; carbendazim, B; benomyl, C; thiophanate-methyl

(mean 68.6 mm) and sensitive (mean 66.5 mm) isolates, but the growth of isolates was significantly ( $P=0.05$ ) different between isolates regardless of resistance or sensitivity. The mean mycelial growth of resistant isolates was 3.2% faster than that of sensitive (Table 1).

**Sporulation and spore germination.** There were significant ( $P=0.05$ ) variations in sporulation among the isolates regardless of resistance or sensitivity to fungicides. However, sporulation of carbendazim-resistant and sensitive isolates on the fungicide-free medium were not different ( $P=0.05$ ) (Table 1). Carbendazim-sensitive isolates, K745 and K765, produced more spores than the other isolates, but on fungicide-free pears, spore production rates of fungicide-resistant isolates were similar to those of sensitive isolates (Table 1).

In fungicide-free potato dextrose broth (PDB), all isolates showed more than 89% of spore germination rates (Table 1). The germination rates were not significantly different ( $P=0.05$ ) from each other.

**Virulence.** The durations of incubation period (time from inoculation to initial symptom expression) and latent period (time from inoculation to sporulation) on pear were very similar between carbendazim-resistant and sensitive isolates (Table 2). The incubation period of carbendazim-resistant isolates ranged from 27.3 to 29.5 hours (mean 28.1 hours). However, incubation periods of carbendazim-sensi-

**Table 1.** Mycelial growth, sporulation, and germination of benzimidazole-resistant and sensitive isolates of *M. fructicola* isolated from peach orchards in Korea<sup>a</sup>

Isolates	Mycelial growth on PDA		Sporulation on		Spore germination (%) <sup>c</sup>
	Rank	Diameter (mm)	PDA ( $\times 10^4$ spores)	Pear <sup>b</sup>	
<b>BS<sup>d</sup></b>					
D758	1	73.0 x	2.6 c	++	90.2 a
K745	5	68.2 xy	8.5 a	+++	90.0 a
K765	8	56.1 z	10.5 a	+++	91.2 a
K7106	4	68.5 xy	3.3 c	++	90.3 a
<b>BR<sup>e</sup></b>					
D795	6	67.0 yz	4.7 b	++	90.2 a
K726	3	72.5 x	5.6 b	+++	90.3 a
K740	7	62.3 z	4.5 bc	++	89.7 a
K760	2	72.4 xy	3.6 bc	++	90.1 a

<sup>a</sup>Means with the same letter in the column are not significantly different at  $P=0.05$  in Duncan's multiple range test.

<sup>b</sup>++; rare, +++; abundant.

<sup>c</sup>Spore germination was examined with 300 spores after 8 hr incubation.

<sup>d</sup>BS represents benzimidazole-sensitivity.

<sup>e</sup>BR represents benzimidazole-resistance.

tive isolates (K745 and K765) were shorter than those of the other isolates (Table 2). There were significant differences ( $P=0.05$ ) in incubation time between isolates. On the other hand, latent periods of carbendazim-resistant isolates ranged from 44.8 to 45.3 hr (mean 45.1 hr), and no significant difference ( $P=0.05$ ) was observed among the isolates.

Lesion diameter of carbendazim-sensitive isolate K7106

**Table 2.** Virulence of benzimidazole-resistant and sensitive *M. fructicola* isolates on pears<sup>a</sup>

Isolates	Incubation period (hr) <sup>b</sup>	Latent period (hr) <sup>c</sup>	Lesion diameters (mm)
<b>BS<sup>d</sup></b>			
D758	29.0 x	44.8 x	55.8 x
K745	26.0 y	44.8 x	51.7 x
K765	26.8 y	43.9 x	53.7 x
K7106	28.3 x	44.6 x	48.0 x
<b>BR<sup>e</sup></b>			
D795	27.3 y	45.3 x	59.4 x
K726	28.4 x	45.3 x	53.6 x
K740	29.5 x	44.8 x	55.2 x
K760	27.3 y	44.9 x	55.2 x

<sup>a</sup>Means with the same letter in the column are not significantly different at  $P=0.05$  in Duncan's multiple range test

<sup>b</sup>Time span from inoculation to initial symptom expression

<sup>c</sup>Time span from inoculation to sporulation from the lesion surface on pear.

<sup>d</sup>Benzimidazole-sensitive.

<sup>e</sup>Benzimidazole-resistant

was small, but the diameter was not significantly different from that of carbendazim-resistant isolates (Table 2).

**EC<sub>50</sub> values in subculture condition.** The initial EC<sub>50</sub> values of carbendazim to *M. fructicola* isolate were between 0.002 and 938.7 µg a.i./ml (Table 3). Among the 4 benzimidazole-sensitive isolates, sensitivity of D758 and K745 to carbendazim was not changed significantly ( $P=0.05$ ) even after 16 times of consecutive subcultures on fungicide-free PDA (Table 3). On the other hand, the carbendazim sensitivity increased right after the first subculture in K765 and K7106. Also, EC<sub>50</sub> values on the two isolates, K726 and K7106, were not changed significantly ( $P=0.05$ ) after 16 times of consecutive subcultures on fungicide-free PDA. While the other two resistant isolates, D795 and K760, showed significant decrease of ( $P=0.05$ ) EC<sub>50</sub> values after 12 times of subcultures (Table 3).

**Stability in storage.** After the storage for 16 weeks at 4°C, the EC<sub>50</sub> values of D795 and K740 decreased significantly ( $P=0.05$ ), compared to the initial EC<sub>50</sub> values (Fig. 3), but K726 and K760 isolates show any significant changes of EC<sub>50</sub> values (Fig. 3).

**Competitive ability.** The competitive ability test was conducted on canned peach with K726 (carbendazim-resistant) and K765 (carbendazim-sensitive). In all mixture pairs, resistance rates of reisolated spores were similar to initial inoculation (Fig. 4). In all comparisons on fruit, the competitive ability of resistant isolates were not significantly ( $P=0.05$ ) different from that of sensitive isolates (Fig. 4).

**Table 3.** Changes in EC<sub>50</sub> of benzimidazole-resistant and sensitive *M. fructicola* isolates to carbendazim by subculturing<sup>a,b</sup>

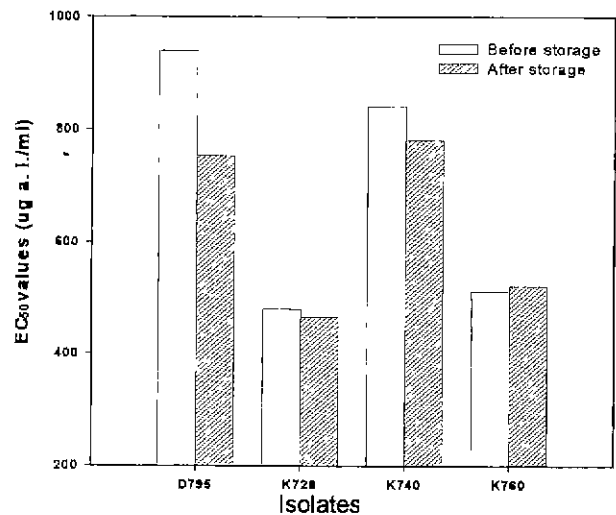
Isolates	Number of subculture				
	0	1	2	3	4
<b>BS<sup>c</sup></b>					
D758	0.015 x	0.075 x	0.013 x	0.018 x	0.010 x
K745	0.002 x	0.002 x	0.007 x	0.007 x	0.009 x
K765	0.123 x	0.014 y	0.010 y	0.011 y	0.009 y
K7106	0.109 x	0.042 y	0.038 y	0.039 y	0.016 y
<b>BR<sup>d</sup></b>					
D795	938.7 x	900.1 x	902.8 x	892.0 x	463.8 y
K726	479.6 x	450.0 x	448.2 x	448.9 x	416.0 x
K740	841.0 x	810.1 x	790.0 x	790.3 x	607.1 y
K760	511.3 x	490.0 x	480.6 x	481.2 x	434.7 x

<sup>a</sup> Isolates were subcultured on fungicide-free PDA at 7 days interval and dose responses was test on PDA amended with 0.01, 0.1, 1, 10, 100, and 1000 µg a.i./ml of carbendazim.

<sup>b</sup> Means with the same letter in the row are not significantly different at  $P=0.05$  in Duncan's multiple range test.

<sup>c</sup> Benzimidazole-sensitivity.

<sup>d</sup> Benzimidazole-resistance.

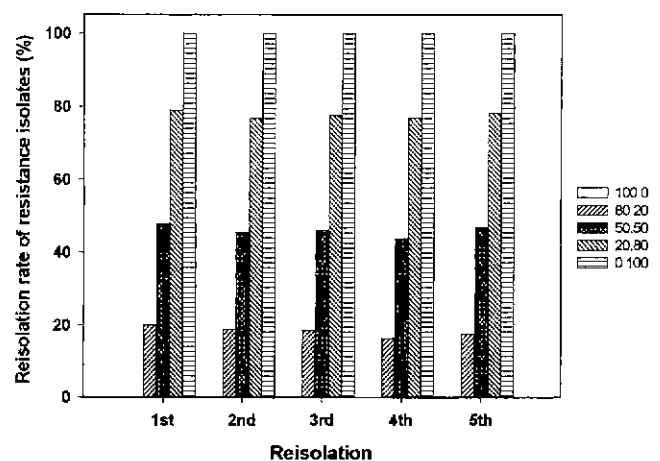


**Fig. 3.** Stability of carbendazim-resistant *M. fructicola* isolates after storing for 16 weeks at 4°C. After 16 weeks, all isolates were grown on fungicide-free PDA for 7 days and then cultured on PDA amended with 0.01, 0.1, 1.0, 10, 100, and 1000 µg a.i./ml of carbendazim for resistance test

## Discussion

Relative growth indices of carbendazim-resistant isolates on PDA amended with carbendazim ranged from 0.6 to 1.0 at the concentration of 1.0 µg a.i./ml, but were smaller than 0.38 at the concentration of 1000 µg a.i./ml. All of those isolates had bigger relative diameter indices of the colonies (0.46) at 100 µg a.i./ml of benomyl or thiophanate-methyl (Fig. 2).

On the other hand, the indices of all the sensitive isolates remained 0 at the different doses of carbendazim (from 1.0



**Fig. 4.** Reisolation rates of benzimidazole-resistant and sensitive *M. fructicola* isolates from peach inoculated with suspensions of various conidial concentrations of resistant and sensitive isolates. Responses to fungicide were tested on PDA amended with 1.0 µg a.i./ml of carbendazim. Symbols represent mixing ratio of spores for benzimidazole-sensitive and resistant isolates.

to 1000 µg a.i./ml) (Fig. 2A). The index ranges of the sensitive isolates were similar to that of *Didymella bryoniae* which is sensitive to benomyl and thiophanate-methyl (Keinath and Zitter, 1994).

Carbendazim was more inhibitory to *M. fructicola* than benomyl and thiophanate-methyl. For example, the indices of the sensitive isolates were 0 at 0.1 µg a.i./ml of carbendazim, but ranged from 0.7 to 1.0 at 0.1 µg a.i./ml of benomyl and from 0.83 to 1.0 on the medium amended with thiophanate-methyl (Fig. 2).

The fitness compositions of resistant isolates including mycelial growth on fungicide free medium, sporulation on fungicide-free medium and pear fruits, and their virulence to pear fruits were similar to those of sensitive isolates (Tables 1 and 2). The virulence, sporulation, and lesion size on pear of resistant-isolates suggests that there is a potential for dominance in the population and selection pressure generated by benzimidazole fungicides may cause difficulty in disease control at the regions of low resistant density in Korea as reported by Lim et al. (1998).

In this study and based on literature review (Delp, 1988), the fitness of the resistant isolates seemed to be same as that of the sensitive isolates. In addition, the isolates resistant to carbendazim persisted for a long time under the experimental conditions (16 times of subculture and storage at 4°C for 16 weeks), which was similar to the result of *Botrytis cinerea* (Delp, 1988).

After inoculation with the mixture of spore suspensions of resistant- and sensitive-isolates, the re-isolation rate of the resistant spores was not significantly ( $P=0.05$ ) reduced regardless of their mixing ratios. From the results, it could be concluded that the competitive ability of the resistant isolates obtained from peach orchards in Korea was similar to that of the sensitive isolates. Therefore, in the regions of low carbendazim-resistant fungal populations such as Chochiwon and Youngduk in Korea reported by Lim et al. (1998), the selection pressure generated by benzimidazole fungicides and the incidence of isolates resistant to carbendazim in the field population of *M. fructicola* are likely to be increased (Sanamuang and Gaunt, 1995).

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