Activity of Essential Oils Against *Bacillus subtilis* Spores

Lawrence, Hayley A. and Enzo A. Palombo*

Environment and Biotechnology Centre, Faculty of Life and Social Sciences, Swinburne University of Technology, Hawthorn, Victoria 3122, Australia

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Alternative methods for controlling bacterial endospore contamination are desired in a range of industries and applications. Attention has recently turned to natural products, such as essential oils, which have sporicidal activity. In this study, a selection of essential oils was investigated to identify those with activity against *Bacillus subtilis* spores. Spores were exposed to 13 essential oils, and surviving spores were enumerated. Cardamom, tea tree, and juniper leaf oils were the most effective, reducing the number of viable spores by 3 logs at concentrations above 1%. Sporicidal activity was enhanced at high temperatures (60°C) or longer exposure times (up to 1 week). Gas chromatography–mass spectrometry analysis identified the components of the active essential oils. However, none of the major oil components exhibited equivalent activity to the whole oils. The fact that oil components, either alone or in combination, did not show the same level of sporicidal activity as the complete oils suggested that minor components may be involved, or that these act synergistically with major components.

Scanning electron microscopy was used to examine spores after exposure to essential oils and suggested that leakage of spore contents was the likely mode of sporicidal action. Our data have shown that essential oils exert sporicidal activity and may be useful in applications where bacterial spore reduction is desired.

**Keywords:** *Bacillus subtilis*, endospores, essential oils, sporicidal, scanning electron microscopy

Bacterial contamination is a concern in a range of industries, including food and pharmaceutical production, and medical environments. Although sterilization can be achieved by heat, chemical, and UV irradiation treatments, these can be impractical when dealing with food products, compromising quality, taste, nutrition, and other properties important to the consumer [21]. In some cases, bacterial contamination is accompanied by the production of endospores, highly resistant cells that can remain dormant until favourable conditions are encountered [24]. Bacteria that produce endospores can prove very difficult to eradicate, and can require high concentrations of chemicals or heat treatments that can destroy the integrity of the product [21, 26]. This problem has inspired research into naturally occurring products with antibacterial properties that may be able to replace or work in conjunction with current methods of eliminating contamination. In particular, plant-derived compounds and essential oils, used for centuries in food and medicine, are being widely investigated for their action against bacteria and spores [4].

A range of chemical agents has been used to exert sporicidal effects. It has been shown that some chemical treatments kill via damage to DNA and additionally cause survivors of chemical treatment to show higher mutation rates, decreasing the chance of successful outgrowth [24]. Chemical treatments can also cause spore death via inactivation of various stages of the germination process, or simply irreversibly disrupt spore integrity [26]. Glutaraldehyde and formaldehyde exhibit particularly favorable sporicidal activity, and have been widely investigated [21]. However, concerns about using glutaraldehyde involve the high concentrations required and extensive contact time to observe significant log reductions in spore numbers. For more resistant spore-forming species, including *Bacillus subtilis*, contact time has been shown to be up to 10 h for a complete kill. Additionally, the pungent odorous properties make glutaraldehyde inappropriate for frequent human mediated use, and in applications such as food sterilization [21]. Formaldehyde is sporicidal at a slower rate compared with glutaraldehyde, and can be used in both liquid and gaseous phases. Unfortunately, the latter phase appears to be more effective, which restricts its use [21]. Chlorine dioxide and hypochlorite are also chemicals used in combating spore populations. Chlorine gas, although effective, is too
hazardous for general use [21]. Hydrogen peroxide has
long been utilized as a sporidial agent; however, to be
effective, it requires high concentrations [21], long contact
time periods [1], needs to be used in conjunction with high
temperatures [22], and additionally tends to be unstable
[21]. It has also been proposed that spores are capable of
forming protective clumps and producing a catalase to
destroy hydrogen peroxide [20].

UV and microwave radiations have additionally been
shown to be as equally effective as conventional heating in
killing Bacillus spores. Microwave radiation is particularly
attractive owing to its commercial availability and low cost
[5, 24]. Sterilization via plasma discharges is also being
investigated and may be applied to sterilization of medical
devices and implants [2]. However, these treatments are
not applicable to the food industry and require extreme
cautions if used around humans.

Owing to the limitations of current sporicidal treatments,
focus has turned to natural products that can produce the
same effect. Such products include essential oils, which
are the volatile oils obtained from plant material, retaining
characteristic properties of the plant useful for applications
in perfume, flavoring, and pharmaceuticals [4]. Importantly,
essential oils exert antibacterial, antiviral, antifungal,
antimycotic, antitoxigenic, antiparasitic, and insecticidal
properties and are considered commercially acceptable for
use in food products, being derived from natural products,
and often accompanied by a pleasant fragrance [4]. Their
characteristics differ according to their content of major
compounds (e.g., terpenes, sesquiterpenes, aldehydes, ketones,
phenols, esters, ethers, and oxides).

Whereas the antimicrobial properties of plant-derived
chemicals and essential oils from numerous plant species
have been well documented against vegetative cells [10,
15], the activity against endospores has not been widely
investigated. However, recent studies have indicated that
natural products, such as green tea catechins [13] and
polyphenols [23], and extracts of the exotic fruit Torilis
japonica, exert activity against endospores [7, 8].

When essential oil components thymol and carvacrol
were used in conjunction with heat treatment, the lag phase
of Bacillus megaterium was increased and growth was
inhibited for a longer time compared with the effect of heat
treatment on its own [19]. These tests were performed in a
carrot broth growth medium in order to investigate the
potential use of these products as food preservatives.
Hernandez-Herrero et al. [14] also utilized carrot-broth-
based growth media to simulate the conditions of minimally
processed foods. They tested the sporidial activity of
essential oils and components against Bacillus cereus
spores at low temperatures. It was found that cinnamon
essential oil and its major component, cinnamaldehyde,
causd complete inhibition of growth at 0.005% (v/v),
suggesting that their use in combination with refrigeration
may be valuable for processed foods. Hence, essential oils
can enhance conventional sporidial treatments.

In the present study, the ability of a selection of commercial
essential oils to reduce the viability of Bacillus subtilis
spores was investigated. Chemical analysis was used to
identify the major components of active essential oils, and
pure components common to oils exhibiting sporidial
activity were tested to determine how they contribute to
the overall activity of the essential oil. Finally, SEM was
used to visualize morphological changes in treated spores.

**Materials and Methods**

**Essential Oils and Components**

Thirteen essential oils used were supplied by Pure Oils (Arundel,
Queensland, Australia). These included Bergamot (Citrus bergamis),
Cardamom (Elettaria cardamomum), Clove Bud (Syzgium aromaticum),
Eucalyptus Blue Gum (Eucalyptus globulus), Juniper Leaf (Juniperus
communis), Laurel Leaf (Laurus nobilis), Lemongrass (Cymbopogon
flexuosus), Palmarosa (Cymbopogon martini), Peppermint (Piper
niagrum), Pine (Pinus pinaster), Tea Tree (Melaleuca alternifolia),
Thyme (Thymus vulgaris), and Yarrow (Achillea millefolium).
All oils were extracted by steam distillation, except Bergamot, which
was cold pressed. A second collection of essential oils was supplied
by New Directions (Sydney, New South Wales, Australia). These
included Tea Tree, Cardamom, and Juniper Leaf. All oils were
extracted by steam distillation. Major essential oil components
including α-terpinyl acetate (86487, Fluka >90% GC), terpinene-4-
ol (86477, Fluka, 98.5% GC), and 1,8-Cineole (C80601, Fluka, 99%
GC) were supplied by Sigma-Aldrich (Castle Hill, Australia).

**Preparation of Spore Suspension**

Essential oils were tested against spores of Bacillus subtilis
( ATCC 6051). Microbiological media were supplied by Oxoid Ltd (Basingstoke,
England). Spore suspensions were prepared as previously described
[14, 16, 18, 19, 23, 25] with minor modifications. Bacteria were grown
on nutrient agar (NA) plates for 1 week to induce sporulation. Spores
were then washed from the plates using 2 ml of sterile H2O,
and transferred to a 50-ml centrifuge tube. The suspension was centrifuged
at 4,500 ×g for 10 min and washed with sterile H2O twice to
remove debris. The final spore pellet was resuspended in 2 ml of
sterile H2O and heated at 80°C for 20 min to kill vegetative cells.
Spore suspensions were stored at 4°C. The presence and purity
(>95%) of spores were confirmed by microscopic examination after
spore staining and the number of viable spores was determined using
a viable plate count.

**Determination of Sporicidal Activity**

Five ml of spore suspension (approximately 2×10^6 CFU/ml) was
mixed with 50 μl of essential oils (or components) and incubated at
various temperatures and periods of time. After treatment, spores
were recovered by centrifugation ( 14,500 ×g for 5 min) and washed
in sterile saline three times to avoid the effects of residual essential
oils on the growth of vegetative cells [8]. The resulting spore pellet
was resuspended in 1 ml of saline, serially diluted, and plated onto
NA to determine the number of colony forming units. To determine
the minimum sporidial concentration, essential oils were diluted to
Sporicidal Activity of Essential Oils
Preliminary screening of the first collection of 13 essential oils indicated that all oils exhibited some level of sporicidal activity after 24 h (Table 1). However, three of the essential oils, cardamom, juniper leaf, and tea tree, consistently showed the greatest level of activity, reducing the number of viable spores by approximately 3 log units. To determine whether the acidity of the essential oils was responsible for their activities, the pH of all oils was determined. Some oils were acidic (pH 4 or 5), whereas others were more neutral (pH 6). To eliminate low pH as the sporicidal factor, spores were incubated at 37°C for 24 h in a sodium acetate–acetic acid buffer adjusted to pH 4. The pH-adjusted spore solution showed no notable reduction in viable spores compared with a pH-neutral control (data not shown), indicating that the spores were acid tolerant and that essential oil components, not their acidic nature, were responsible for the observed sporicidal effects. Further experiments were performed only with the three most active essential oils. These essential oils produced by an alternative supplier were also investigated and shown to have comparable sporicidal activity.

Effects of Temperature and Time on Sporicidal Activity
The sporicidal activities of the three selected oils were investigated over a range of temperatures (4°C, 37°C, and 60°C) and time exposures (24 h, 48 h, and 1 week) (Table 2). These experiments indicated that exposure to temperatures above 37°C enhanced the activity of some oils, particularly tea tree oil. Similarly, extended exposure of spores for up to a week enhanced the activity of all oils, with the sporicidal activity of cardamom oil increasing by almost 3 logs. This enhanced activity was not due to a pH change, as the oils only varied by 0.5 pH units when measured at 4°C, 37°C, and 60°C. At shorter exposure times (2 h), considerable sporicidal activity was also apparent with approximately 2 log reductions in viability (data not shown).

Determination of Minimum Sporicidal Concentrations
Essential oils in their pure form can be considered irritants for human use. Therefore, it was useful to know if their sporicidal effectiveness persisted after dilution. A series of dilutions of cardamom, juniper leaf, and tea tree oils was

Table 1. Sporicidal activity of essential oils.

<table>
<thead>
<tr>
<th>Essential oil</th>
<th>Log reduction in viable spores at 4°C</th>
<th>Log reduction in viable spores at 37°C</th>
<th>Log reduction in viable spores at 60°C</th>
<th>Log reduction in viable spores at 24 h</th>
<th>Log reduction in viable spores at 48 h</th>
<th>Log reduction in viable spores at 1 week</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bergamot</td>
<td>2.47±0.21</td>
<td>3.12±0.21</td>
<td>1.68±0.21</td>
<td>3.05±0.21</td>
<td>3.59±0.21</td>
<td>4.90±0.21</td>
</tr>
<tr>
<td>Cardamom</td>
<td>2.62±0.18</td>
<td>3.20±0.18</td>
<td>2.74±0.18</td>
<td>3.47±0.21</td>
<td>3.95±0.21</td>
<td>4.80±0.21</td>
</tr>
<tr>
<td>Clove Bud</td>
<td>2.40±0.18</td>
<td>3.05±0.18</td>
<td>2.90±0.18</td>
<td>3.40±0.21</td>
<td>3.90±0.21</td>
<td>4.80±0.21</td>
</tr>
<tr>
<td>Eucalyptus Blue Gum</td>
<td>2.40±0.18</td>
<td>3.05±0.18</td>
<td>2.90±0.18</td>
<td>3.40±0.21</td>
<td>3.90±0.21</td>
<td>4.80±0.21</td>
</tr>
<tr>
<td>Juniper Leaf</td>
<td>2.40±0.18</td>
<td>3.05±0.18</td>
<td>2.90±0.18</td>
<td>3.40±0.21</td>
<td>3.90±0.21</td>
<td>4.80±0.21</td>
</tr>
<tr>
<td>Laurel Leaf</td>
<td>2.40±0.18</td>
<td>3.05±0.18</td>
<td>2.90±0.18</td>
<td>3.40±0.21</td>
<td>3.90±0.21</td>
<td>4.80±0.21</td>
</tr>
<tr>
<td>Lemongrass</td>
<td>2.40±0.18</td>
<td>3.05±0.18</td>
<td>2.90±0.18</td>
<td>3.40±0.21</td>
<td>3.90±0.21</td>
<td>4.80±0.21</td>
</tr>
<tr>
<td>Palmarosa</td>
<td>2.40±0.18</td>
<td>3.05±0.18</td>
<td>2.90±0.18</td>
<td>3.40±0.21</td>
<td>3.90±0.21</td>
<td>4.80±0.21</td>
</tr>
<tr>
<td>Peppermint</td>
<td>2.40±0.18</td>
<td>3.05±0.18</td>
<td>2.90±0.18</td>
<td>3.40±0.21</td>
<td>3.90±0.21</td>
<td>4.80±0.21</td>
</tr>
<tr>
<td>Pine</td>
<td>2.40±0.18</td>
<td>3.05±0.18</td>
<td>2.90±0.18</td>
<td>3.40±0.21</td>
<td>3.90±0.21</td>
<td>4.80±0.21</td>
</tr>
<tr>
<td>Tea Tree</td>
<td>2.40±0.18</td>
<td>3.05±0.18</td>
<td>2.90±0.18</td>
<td>3.40±0.21</td>
<td>3.90±0.21</td>
<td>4.80±0.21</td>
</tr>
<tr>
<td>Yarrow</td>
<td>2.40±0.18</td>
<td>3.05±0.18</td>
<td>2.90±0.18</td>
<td>3.40±0.21</td>
<td>3.90±0.21</td>
<td>4.80±0.21</td>
</tr>
</tbody>
</table>

*Average and standard deviations of triplicate experiments.

Table 2. Effects of temperature and incubation time on sporicidal activity.

<table>
<thead>
<tr>
<th>Essential oil</th>
<th>4°C</th>
<th>37°C</th>
<th>60°C</th>
<th>24 h</th>
<th>48 h</th>
<th>1 week</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cardamom</td>
<td>4.26±0.09</td>
<td>3.19±0.02</td>
<td>3.95±0.30</td>
<td>3.12±0.02</td>
<td>3.95±0.79</td>
<td>6.04±0.63</td>
</tr>
<tr>
<td>Juniper Leaf</td>
<td>2.69±0.01</td>
<td>2.62±0.28</td>
<td>4.26±0.34</td>
<td>2.74±0.28</td>
<td>3.65±0.10</td>
<td>3.56±0.76</td>
</tr>
<tr>
<td>Tea Tree</td>
<td>2.63±0.24</td>
<td>3.63±0.41</td>
<td>6.26±0.34</td>
<td>3.05±0.41</td>
<td>4.08±0.39</td>
<td>4.04±0.81</td>
</tr>
</tbody>
</table>

*Temperature experiments performed for 24 h; time experiments were performed at 37°C; averages and standard deviations of triplicate experiments.
tested against *B. subtilis* spores. The sporicidal effectiveness was such that at levels above 10% (v/v), equivalent sporicidal activity was observed. At levels below 10% (v/v), a sharp decrease in activity was observed. At 1% (v/v) concentration, treated spores displayed no reduction in viability compared with the control. The minimum sporicidal concentration was therefore identified as >1% (v/v). The viability of spores was not affected after treatment with DMSO (solvent-only control).

**Sporicidal Activity of Essential Oil Components**

Previous studies of the role that individual components of essential oils play in antibacterial activity have been somewhat contradictory, suggesting that in some cases pure components of the oil seem to have equal antibacterial qualities as the oil itself, whereas in others the pure oil, with all major and minor components working synergistically, showed superior action [3, 6]. GC–MS was therefore utilized to identify the components in the active essential oil samples. Products from both manufacturers were analyzed.

GC–MS analysis identified three major components (terpinene-4-ol, α-terpinyl acetate, and 1,8-cineole) present in the two most effective essential oils, namely tea tree oil and cardamom oil (Table 3). The presence of these components was verified by comparative analysis of authentic samples. It is of interest to note that these three components were not present in any of the less active essential oils in any abundance. From Table 4, it can be seen that the selected components reduced viable spores by approximately 2 log units. In no case did the components, alone or in combination (in the same proportion as found naturally in the essential oils), exhibit sporicidal effects comparable to the level of inhibition seen with complete oils. It is particularly interesting that the combination of α-terpinyl acetate and 1,8-cineole did not exhibit higher sporicidal activity considering that these components comprised approximately 80% of the cardamom essential oil used in this study. Other combinations of oil components (1:1 blends) were also tested and exhibited similar sporicidal effects to those reported in Table 4.

**Table 4. Sporicidal activity of essential oil components.**

<table>
<thead>
<tr>
<th>Essential oil component(s)</th>
<th>Log reduction in viable spores$^*$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Terpinen-4-ol</td>
<td>0.77±0.59</td>
</tr>
<tr>
<td>α-Terpinyl acetate</td>
<td>1.79±0.58</td>
</tr>
<tr>
<td>1,8-Cineole</td>
<td>2.28±0.18</td>
</tr>
<tr>
<td>Terpinen-4-ol+α-terpinyl acetate</td>
<td>1.92±0.41</td>
</tr>
<tr>
<td>1,8-Cineole+α-terpinyl acetate</td>
<td>2.08±0.28</td>
</tr>
</tbody>
</table>

$^*$Experiments performed at 37°C for 24 h; averages and standard deviations of triplicate experiments.

**Scanning Electron Microscopy (SEM)**

SEM was utilized to visualize the effect of selected essential oils on the morphology of *B. subtilis* spores (Fig. 1). Untreated spores were plump and oval, with slight veiny ridges. However, treated spores appeared withered and deflated with pronounced ridges. This was apparent for spores treated for 4 h (data not shown), but was particularly

**Table 3. Composition (%) of cardamom and tea tree oils used in this study**

<table>
<thead>
<tr>
<th>Compound</th>
<th>Cardamom oil</th>
<th>Tea tree oil</th>
</tr>
</thead>
<tbody>
<tr>
<td>1,8-Cineole$^b$</td>
<td>34.0</td>
<td>5.5</td>
</tr>
<tr>
<td>Limonene</td>
<td>2.2</td>
<td>1.1</td>
</tr>
<tr>
<td>Longifolene</td>
<td>–</td>
<td>1.9</td>
</tr>
<tr>
<td>Sabinen hydrate acetate</td>
<td>3.0</td>
<td>–</td>
</tr>
<tr>
<td>Sabine</td>
<td>2.9</td>
<td>–</td>
</tr>
<tr>
<td>Terpinen-4-ol$^b$</td>
<td>–</td>
<td>38.0</td>
</tr>
<tr>
<td>Terpinolene</td>
<td>–</td>
<td>19.3</td>
</tr>
<tr>
<td>Viridiflorene</td>
<td>–</td>
<td>1.7</td>
</tr>
<tr>
<td>α-Terpinene</td>
<td>–</td>
<td>10.0</td>
</tr>
<tr>
<td>α-Terpineol</td>
<td>1.1</td>
<td>2.9</td>
</tr>
<tr>
<td>α-Terpinyl acetate$^b$</td>
<td>46.0</td>
<td>–</td>
</tr>
<tr>
<td>δ-Cadinene</td>
<td>–</td>
<td>1.7</td>
</tr>
<tr>
<td>γ-Terpinene</td>
<td>1.5</td>
<td>2.9</td>
</tr>
</tbody>
</table>

$^a$Only compounds present at 1% or greater are listed.

$^b$Verified by comparative GC–MS analysis of authentic samples.

**Fig. 1.** SEM analysis of *B. subtilis* spores treated with tea tree oil (A), cardamom oil (B), or juniper leaf oil (C) for 24 h at 37°C. Untreated spores are in panel (D) and spores treated with DMSO are in panel (E). Scale bars indicate 1 µm in (A), (D), and (E) and 200 nm in (B) and (C).
exaggerated after 24 h. It is interesting to note that juniper leaf-oil-treated spores appeared to be less severely affected compared with those treated with tea tree oil or cardamom oil, supporting the observation that juniper leaf oil had less sporicidal activity (Table 1). Spores treated with DMSO were indistinguishable from untreated spores (Fig. 1), supporting the observations made above that the solvent did not affect the viability of spores.

**DISCUSSION**

In this study, several commercial essential oils were investigated for their abilities to reduce the viability of *Bacillus subtilis* spores. Three oils, cardamom, juniper leaf, and tea tree oils, exhibited greatest activity. The sporicidal activities appeared to be temperature and time dependent, which was particularly apparent for tea tree and cardamom oils. When the major components of the most active oils were tested individually or in combination, the sporicidal activity was much less than any of the complete oils. Our results support earlier work that showed that formulations containing 5–15% tea tree oil were able to reduce the number of viable *Bacillus* spores [17].

One proposed application of essential oils in the food industry is their addition to packaged food products as a means of extending shelf life and further preventing spoilage. It has previously been demonstrated that the addition of essential oils, or their components, enhanced the effects of heat treatment on food products [14, 19]. If essential oils are increasingly sporicidal at increased temperatures, this could make them ideal additions to canned foods, given they are heat treated after packaging to kill bacterial contamination, but this treatment can be ineffective in eliminating spores. However, it will be important to determine if the addition of essential oils to foods, even at low concentrations, is associated with changes to the sensory properties (*i.e.*, unpleasant odor or taste) of the foods. Such adverse outcomes could prevent the use of essential oils, or their components in foods, and further work in this area is needed [14].

The effectiveness of essential oils after dilution is beneficial if applied as an addition to general, frequent use, household products, such as disinfectants or to topical skin treatments. Products that are both of natural origin and requiring only low concentrations to be functional are desirable for close continued human contact. The finding that the essential oils retained their sporicidal activity after considerable dilution is an important observation and increases their potential to be used in this context.

One of the most important findings of the current study was the reduced effectiveness of the major components of essential oil compared with whole oil mixtures. Many reports pertaining to the antibacterial activity of essential oil components have tested their efficacy against vegetative bacterial cells [3, 11, 12]. Considering the resilient nature of spores, it is not completely surprising that the same effects have not been replicated in this study, and that the action of one or two components was not equal to that of the mixture of components in the oil. Although the actions of the major components need to be further investigated, it is likely that the antibacterial and sporicidal activities of essential oils are the result of different components. Given our findings, it is possible that some of the minor or trace chemicals play a role in the sporicidal activity of an essential oil. Increasing the number of components tested, individually and in combination, could give a better idea of their contribution to the overall activity of an essential oil.

SEM analysis indicated that exposure to essential oils resulted in visible damage to the spore coat. Although spores have extremely low water content [26], some intracellular material may have been lost from the treated spores, as suggested by their shrivelled, dehydrated morphology. Previous studies have suggested that certain essential oil components are able to interact with membranes and disrupt structure by increasing fluidity [11]. The weakening of the spore membrane structure in this manner could also explain the collapsed morphology of the treated spores. Cortezzo et al. [9] elucidated the mechanism by which a number of essential oil compounds can both reversibly and irreversibly inhibit spore germination. The components interrupt the action of, or response to, nutrient receptors involved in the cascade of changes that lead a spore to commit to germination. Clearly, the modes of action of the essential oils investigated in this study need to be further elucidated.

The observation that the sporicidal activity of essential oils was enhanced by increasing temperature suggests that further research should be aimed at investigating the use of essential oils in combination with current treatments to determine whether this may reduce the exposure time required to successfully sterilize spores using heat, chemical, and UV methods. Similarly, prolonged exposure of spores to oils resulted in enhanced activity. It would be worthwhile testing the less effective oils at longer exposure times to determine if their activities would also improve. Testing could additionally be extended to other spore-forming bacteria that pose threats to the food, pharmaceutical, and medical industries, such as *Clostridium*. Overall, this study has provided clear evidence for the activity of essential oils against spores of *B. subtilis*.

**Acknowledgments**

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References


