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# Effects of a butenolide present in smoke on light-mediated germination of Australian *Asteraceae*

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## Abstract

This study investigated the effects of 3-methyl-2*H*-furo[2,3-*c*]pyran-2-one, a germination active butenolide present in plant-derived smoke, gibberellic acid and smoke water on seeds of Australian *Asteraceae* exposed to different light regimes. Seeds of all species required light, with maximum germination occurring under white light, or light dominated by 640 nm. Compared to untreated seeds, butenolide increased germination of *Angianthus tomentosus*, *Gnephosis tenuissima*, *Myriocephalus guerinae*, *Podolepis canescens* and *Rhodanthe citrina* at suboptimal light wavelengths and in the dark to a level equal to, or greater than, smoke water. Germination of *Erymophyllum glossanthus* and *Gnephosis acicularis* was not promoted by butenolide or smoke water under any light regime. The action of gibberellic acid was compared to that of butenolide for three species (*Angianthus tomentosus*, *Myriocephalus guerinae* and *Podolepis canescens*), and both compounds were found to stimulate germination. This study provides evidence that butenolide can act in a similar fashion as gibberellic acid in promoting seed germination of light-sensitive seeds. The ecological significance of these findings is discussed.

**Keywords: *Asteraceae*, butenolide, gibberellic acid, light, seed germination, smoke**

## Introduction

The germination-promotive effects of smoke have long been recognized (De Lange and Boucher, 1990), and have been reported for a wide range of species native to South Africa (Brown *et al.*, 1993; Brown and Botha, 2004), Australia (Dixon *et al.*, 1995; Roche *et al.*, 1997) and California (Keeley and Fotheringham, 1998), as well as crop (Drewes *et al.*, 1995; Thomas and van Staden, 1995) and weed (Doherty and Cohn, 2000; Adkins and Peters, 2001) species. Recently, a germination-active chemical in plant- and cellulose-derived smoke has been isolated and synthesized (Flematti *et al.*, 2004, 2005). The butenolide, 3-methyl-2*H*-furo[2,3-*c*]pyran-2-one, stimulates germination of a number of key smoke-responsive species and is active at very low concentrations (1 $\mu g\,l^{-1}$), indicating that it is a major promotive compound in smoke (Flematti *et al.*, 2004). Identification of the butenolide provides opportunities to study the mode of action of a novel, naturally occurring, germination stimulant and to better understand the role of smoke in seed germination ecology.

A number of studies have investigated the physiology of smoke-stimulated germination. In particular, smoke influences the manner in which seeds respond to light and gibberellic acid (GA), and appears to affect endogenous gibberellic acid (GA) synthesis and abscisic acid (ABA) content (van Staden *et al.*, 2000). For example, smoke substitutes for red light (640 nm) in the germination of lettuce (*Lactuca sativa* L. cv. Grand Rapids) seeds, promoting germination in the dark or in the presence of far-red (730 nm) light (Drewes *et al.*, 1995; van Staden *et al.*, 1995). This smoke-induced promotion of germination is similar to that achieved by treating seeds with $GA_3$ (van Staden *et al.*, 1995), and can be inhibited by exposure to gibberellin biosynthesis inhibitors, such as paclobutrazol (Gardner *et al.*, 2001). The similarity of the smoke and $GA_3$ responses, and the interactions between smoke and other phytohormones in seeds has led to the hypotheses that altered synthesis/metabolism of endogenous hormones, and/or increased sensitivity

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to hormones, are possible modes of action of smoke (van Staden *et al.*, 2000). Indeed, elevated $GA_3$ content in lettuce seeds has been measured after smoke treatment (Gardner *et al.*, 2001), and studies on smoke-responsive *Nicotiana attenuata* seeds (Schwachtje and Baldwin, 2004) have demonstrated that smoke alters the dynamics of endogenous GA and ABA during germination.

Germination studies of smoke-responsive Australian species have generally not focused on interactions with light and/or $GA_3$. However, one study demonstrated that light-requiring seeds of the annual Australian *Asteraceae*, *Shoenia filifolia* subsp. *subulifolia*, germinated in the dark following smoke treatment, and germination (in a 12/12 h light/dark regime) was also increased by exposure to $GA_3$ (Plummer *et al.*, 2001). Numerous other light-stimulated genera of Australian *Asteraceae* also germinate in the dark following treatment with $GA_3$ (Plummer and Bell, 1995), and it is likely that many arid-zone, ephemeral *Asteraceae*, with a light requirement for germination, may respond equally to exogenous application of $GA_3$. Therefore, annual species of Australian *Asteraceae* appear ideal candidates to investigate the action of butenolide on seed germination. In this study, we examined the effects of butenolide on the germination of seeds of seven annual Australian *Asteraceae* in relation to light, $GA_3$ and smoke water.

## Materials and methods

### Seed material

Seven ephemeral *Asteraceae* species, native to the arid zone of Western Australia, were selected for study: *Angianthus tomentosus* J.C. Wendl, *Erymophyllum glossanthus* Paul G. Wilson, *Gnephosis acicularis* Benth., *Gnephosis tenuissima* Cass., *Myriocephalus guerinae* F. Muell., *Podolepis canescens* DC. and *Rhodanthe citrina* (Benth.) Paul G. Wilson. Seeds (technically achenes, but hereafter referred to as seeds) were collected in October and November 2003 from wild plants, air dried and stored under ambient laboratory conditions (*c.* 23°C) until used for experiments, which began in July 2004.

### Experiment 1: Effects of butenolide, smoke water and light regimes on seed germination

For germination tests, seeds were placed in Petri dishes containing Advantec 424 filter paper (Seot Scientific, Perth, Australia) moistened with either de-ionized water (control), butenolide at 100 ppb (0.67 μM) or smoke water solution at 1:10 (v/v) dilution. The butenolide was synthesized as described in Flematti *et al.* (2005). Straw was used to generate the smoke, and smoke water was prepared as described in Dixon *et al.* (1995). Petri dishes were then placed in specially manufactured light-excluding compartments (20 × 20 × 15 cm) with windows made with Kodak Wratten filters (Rokich and Bell, 1995) to expose the seeds to defined wavelength radiation of 570 nm (photo irradiance 0.8 $\mu M\,m^{-2}\,s^{-1}$), 640 nm (photo irradiance 1.24 $\mu M\,m^{-2}\,s^{-1}$), 720 nm (photo irradiance 0.2 $\mu M\,m^{-2}\,s^{-1}$), white light (photo irradiance 15.4 $\mu M\,m^{-2}\,s^{-1}$, 400–700 nm), and continuous darkness. All solutions were applied to the seeds in Petri dishes in complete darkness prior to the Petri dishes being placed in the wavelength box. Petri dishes were rotated in darkness every 30 min for the first 6 h to ensure that all dishes were equally exposed to radiation. All treatments were incubated at 23°C. For *M. guerinae* and *E. glossanthus*, 20 seeds were used for each treatment. For the other species, a roughly equal number of seeds (*c.* 30–50) was placed into each Petri dish, and all seeds were subsequently counted upon scoring to determine percentage germination. All treatments were replicated three times. Germination was scored based on radicle emergence to greater than 2 mm, following 5 d incubation.

### Experiment 2: Comparative effects of butenolide and gibberellic acid on seed germination

Seeds of *A. tomentosus*, *M. guerinae* and *P. canescens* were selected based on the previous experiment demonstrating butenolide-stimulated germination in the absence of light. Seeds were placed in Petri dishes (as described above) moistened with either de-ionized water (control), gibberellic acid ($GA_3$) at 1000 ppm (3 mM), or butenolide at 1, 10 or 100 ppb. All treatments were prepared in complete darkness. The Petri dishes were then incubated at 12/12 h alternating temperature regimes of 18/7°C, 26/13°C and 33/18°C, selected as representative of winter, spring/autumn and summer temperatures likely to be experienced in the habitat range for the study species. Seeds were exposed to a 12/12 h alternating light regime, photo irradiance of 30 $\mu M\,m^{-2}\,s^{-1}$ 400–700 nm, or continuous darkness.

### Statistical analysis

Germination data were analysed for statistical significance by analysis of variance. Percentage values for germination were arcsine transformed prior to analysis (untransformed data appear in all figures). Fisher's least significant difference ($P < 0.05$) was used to determine significant differences between treatments.

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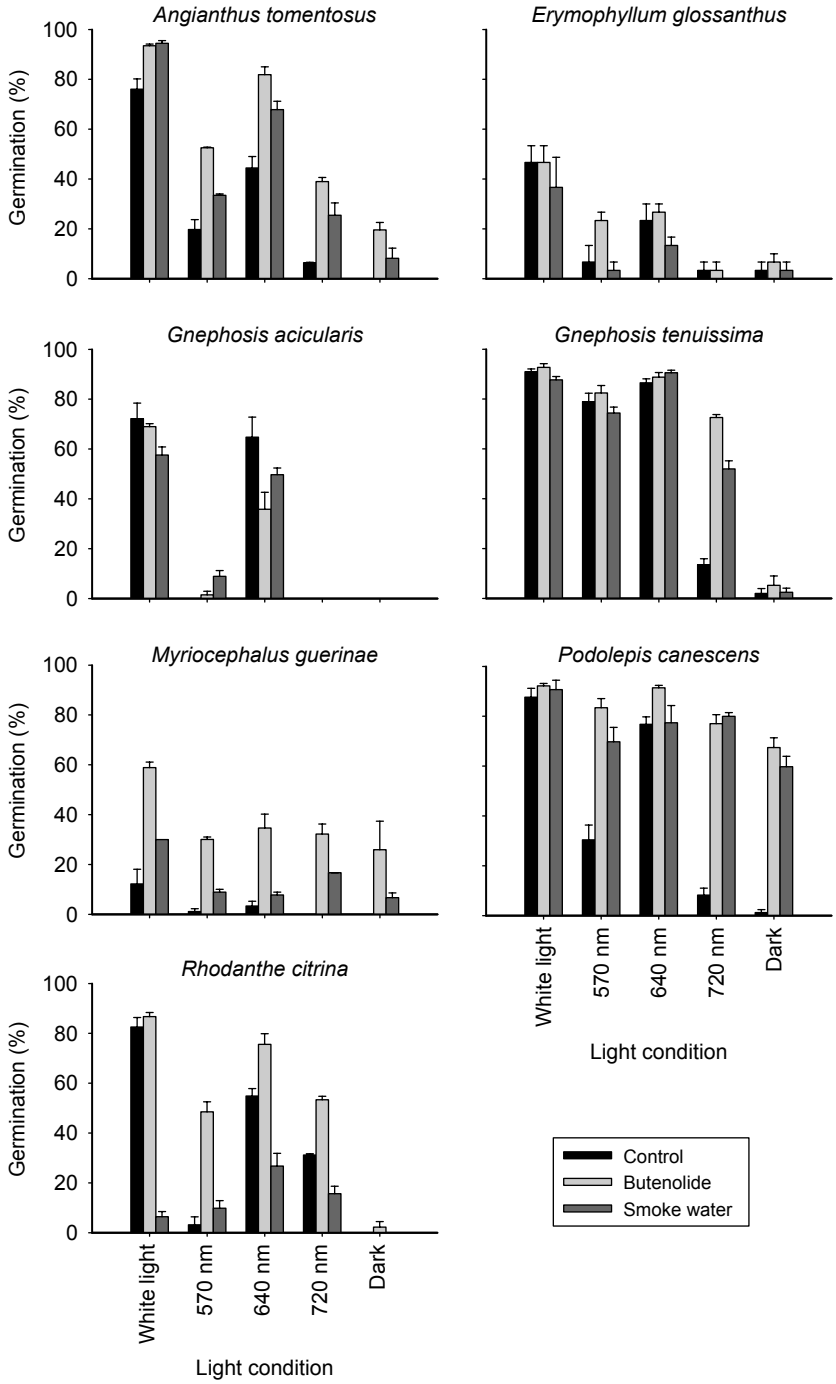


**Figure 1.** Mean percentage germination (± SE) of seven Australian *Asteraceae* species in white light, darkness and over a range of light wavelengths. Seeds were incubated in Petri dishes irrigated with water (control), butenolide (100 ppb) or smoke water (1:10 v/v).

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## Results

### *Effects of butenolide, smoke water and light regimes on seed germination*

For all but one species, germination of untreated control seeds was greatest when exposed to white light, significantly higher ($P < 0.05$) than germination under other light conditions or darkness (Fig. 1). Exposure of seeds to 640 nm produced the next highest germination response, significantly greater ($P < 0.05$) than that of seeds exposed to 570 nm, 720 nm or darkness. Germination was less than 30% under all other light/dark conditions. *G. tenuissima* seeds responded slightly differently compared to the other species, with no difference between germination of seeds exposed to white light or 640 nm, and much higher germination (79%) at 570 nm than in other species.

Compared to control seeds, germination of most species was stimulated by both butenolide and smoke-water, although the wavelengths at which these cues were active varied somewhat between species. *A. tomentosus* and *M. guerinae* seeds were responsive to butenolide and smoke water, with a significant increase ($P < 0.05$) in germination in darkness and across all light regimes (Fig. 1). *M. guerinae* seeds appeared to have an absolute requirement for smoke and/or butenolide, as germination of control seeds was <11%, regardless of light conditions. For both species, germination of butenolide-treated seeds was significantly greater ($P < 0.05$) than that of smoke-water-treated seeds in darkness and all light regimes, except for *A. tomentosus* seeds exposed to white light, where no significant difference was observed between butenolide and smoke water. *R. citrina* seeds responded similarly to butenolide, with a significant increase ($P < 0.05$) in germination at all light regimes, except white light. However, for this species smoke water significantly suppressed germination (compared to control seeds and butenolide-treated seeds) under most light conditions. The promotive effects of butenolide and smoke water were evident as significantly increased germination ($P < 0.05$) of *P. canescens* seeds exposed to 570 nm, 720 nm and darkness and *G. tenuissima* seeds exposed to 720 nm. For the remaining two species, *E. glossanthus* and *G. acicularis*, exposure to smoke water or butenolide did not stimulate germination above that of the controls (except for butenolide-treated *E. glossanthus* seeds exposed to 570 nm).

### *Comparative effects of butenolide and gibberellic acid on seed germination*

For *A. tomentosus* seeds, germination in the light was >80% across all treatments at 26/13°C and 33/18°C (Fig. 2). Nevertheless, a small, but significant increase ($P < 0.05$) in germination, compared to controls, was measured following treatment with butenolide (in most cases) or $GA_3$, and both compounds promoted germination to a similar level at the concentrations tested. For seeds incubated at 18/7°C, germination of control seeds was much less, *c.* 38%, and treatment with 1, 10 or 100 ppb butenolide resulted in significantly greater ($P < 0.05$) germination than either the control seeds or seeds treated with $GA_3$. In the dark, at each of the three temperature regimes, germination of control seeds was negligible, similar to the first series of experiments. Both butenolide and $GA_3$ partially alleviated the light requirement, significantly increasing ($P < 0.05$) germination, but not to the same percentages as seeds incubated in the light. While a few significant differences were noted between germination of seeds exposed to 1, 10 or 100 ppb butenolide or $GA_3$, the germination differences were small, and the incidence of significance was disparate across the treatments.

Similar results occurred for seeds of *P. canescens*. When comparing all treatments, germination percentages were highest at 26/13°C and 33/18°C, and in nearly all cases, both butenolide and $GA_3$ significantly ($P < 0.05$) promoted germination of seeds in the light and dark at all temperatures (Fig. 2). The promotive effects of butenolide and $GA_3$ were again most obvious in dark-incubated seeds.

Germination percentages of seeds of *M. guerinae* were lower than those recorded for the other two species, and the temperature effects on germination were slightly different, with the higher germination percentages recorded at 18/7°C and 26/13°C (Fig. 2). In addition, the differences between seeds germinated in the light and dark were less pronounced than in the other species, although at 18/7°C and 26/13°C, germination of butenolide-treated seeds was still significantly greater ($P < 0.05$) in the light than in the dark in most cases. At all temperatures, germination following $GA_3$ treatment was significantly greater ($P < 0.05$) than control seeds and seeds treated with butenolide. For these seeds, germination percentages generally increased as the concentration of butenolide increased. This concentration gradient response was less evident in the other two species.

## Discussion

For the ephemeral *Asteraceae* tested in this study, in most cases where smoke promoted germination, butenolide likewise promoted germination. This finding suggests that the identified butenolide is the compound in smoke responsible for the enhanced germination, and is consistent with the findings of Flematti *et al.* (2004), who demonstrated that

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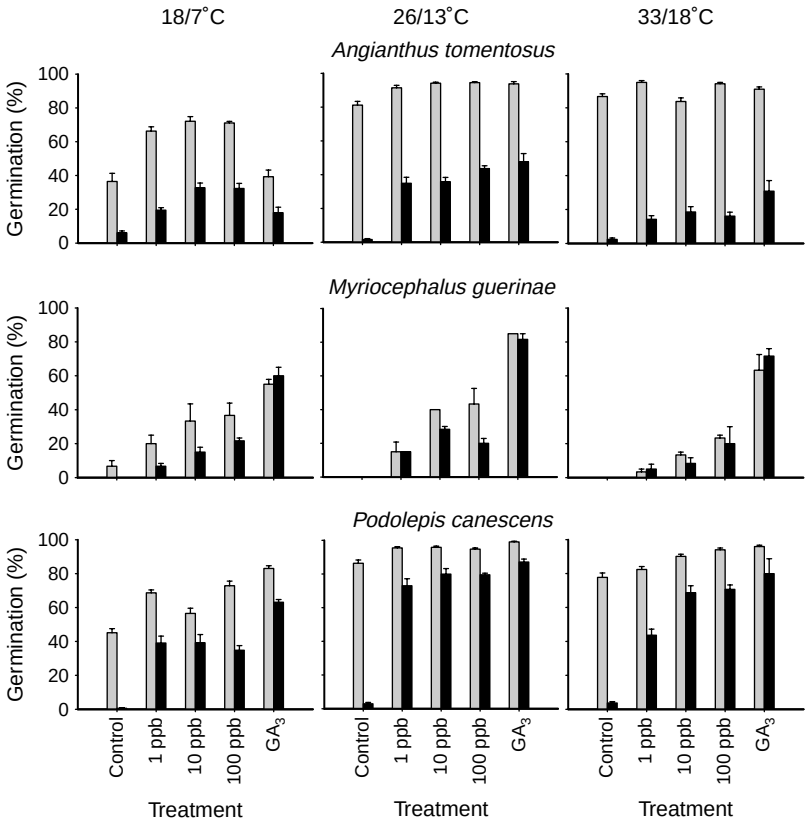


**Figure 2.** Mean percentage germination (± SE) of three Australian *Asteraceae* species in white light (grey bars) or darkness (black bars) at three 12/12 h temperature regimes (18/7°C, 26/13°C and 33/18°C). Seeds were incubated in Petri dishes irrigated with water (control), butenolide at 1 ppb, 10 ppb or 100 ppb, or gibberellic acid ($GA_3$) at 1000 ppm.

butenolide stimulates germination of a wide range of smoke-responsive species. For three species, *A. tomentosus*, *M. guerinae* and *R. citrina*, germination in the presence of butenolide was significantly greater than germination in the presence of smoke water. This increased action of butenolide, compared to smoke water, is possibly attributable to toxic components of smoke water that affect some seeds. Prolonged exposure to smoke water or exposure to high concentrations of smoke water is inhibitory to germination in other species (Drewes *et al.*, 1995), whereas the butenolide retains germination promotion across a very broad range of concentrations [1 ppm, 6.7 μM to <1 ppb, <6.7 nM (Flematti *et al.*, 2004)]. Two species (*E. glossanthus* and *G. acicularis*) did not respond to smoke or butenolide, and it is clear that these species are dependent largely upon other environmental cues to time germination.

Germination of all species was strongly light-dependent, a finding consistent with studies on a number of other genera of ephemeral Australian *Asteraceae* (Plummer and Bell, 1995; Plummer *et al.*, 2001). White light or red light (640 nm) was generally most effective at promoting germination in each species. Germination responses to other wavelengths varied between species; although, with the exception of *G. tenuissima* seeds, <30% germination was recorded in light dominated by 570 nm and 720 nm for all species. This finding conforms to research on a large number of species, demonstrating that the red wavelength component of light is largely responsible for eliciting germination in photoblastic seeds (Casal and Sanchez, 1998).

Exposure to smoke or butenolide partially replaced the light requirement in five species. Further experiments investigating the ability of butenolide and $GA_3$ to replace the light requirement were undertaken on *A. tomentosus*, *M. guerinae* and *P. canescens* seeds. Butenolide was found to promote germination of seeds of these species in a manner similar to $GA_3$. For *M. guerinae* seeds, 1000 ppm $GA_3$ was more effective at promoting germination than 1, 10 or 100 ppb butenolide, but butenolide still significantly increased germination above the controls at all concentrations. For the other two species, broadly comparable results were recorded between each concentration of butenolide and $GA_3$. Germination of photoblastic seeds in the

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dark by exposure to gibberellins is well established, and it is thought that gibberellins, and other phytohormones, are involved in the phytochrome control system that regulates the light response (Thomas, 1992). In this study, the similarity in germination promotion of dark-incubated seeds by butenolide and $GA_3$ conforms to that of other studies investigating smoke- and phytohormone-mediated germination (van Staden *et al.*, 2000), raising the possibility that butenolide may act, at least in part, via moderation of gibberellin metabolism in seeds. Of course, there may be a number of reasons why a portion of a seed lot is receptive to germination stimulants, and the similarity of the response of seeds to $GA_3$ and butenolide does not, on its own, indicate similarity in mechanism of action. However, in both lettuce (van Staden *et al.*, 1995) and *Nicotiana attenuata* (Schwachtje and Baldwin, 2004) seeds, smoke treatment dramatically increased the sensitivity to $GA_3$, and a smoke-induced increase in endogenous $GA_3$ production has also been demonstrated in lettuce seeds (Gardner *et al.*, 2001). For *N. attenuata* seeds, it has been suggested that smoke treatment promotes *de novo* $GA_3$ synthesis (which is possibly linked to light exposure and, thus, phytochrome mediated) (Schwachtje and Baldwin, 2004).

The ecological significance of the response to smoke (*inter alia*, the butenolide) in this study provides intriguing opportunities for understanding the role of disturbance phenomena, such as fire and/or accelerated release of the butenolide through soil via a physical disturbance event. Most of the habitats where the *Asteraceae* study taxa occur are in the arid zone of Western Australia. This harsh environment experiences fires at very low frequency ($\geq$25 years), yet all the study species have highly synchronized germination after episodic, heavy rainfall events (Mott, 1972; Mott and Groves, 1981). After heavy rain the arid zone is transformed with large-scale germination and flowering events over only a few months that carpet many thousands of square kilometres. If the butenolide is released through the action of heavy rainfall, accelerating decay of the accumulated organic matter or some other geochemical activity in soil, this release may be sufficient to provide the cue for germination. Physical disturbance of soil alone is commonly observed to promote germination of Australian native species (Rokich *et al.*, 2002), providing support for the accelerated decay of organic material as a possible source of smoke-like, germination promotive compounds. Biotic production of nitrogen oxides has been suggested as a possible stimulant for non-fire-prone, desert populations of *Emmenanthe penduliflora* (Keeley and Fotheringham, 2000). The biotic production of butenolide now presents an alternative hypothesis, particularly as *E. penduliflora* seeds are highly responsive to butenolide (Flematti *et al.*, 2004), and nitrogen oxides have been eliminated as the germination stimulant in smoke responsible for germination of *E. penduliflora* (Preston *et al.*, 2004). Pierce *et al.* (1995) suggested that smoke-induced germination of seeds of a number of *Mesembryanthemaceae* species from non-fire-prone, arid habitats cast doubt on the ecological significance of smoke as a fire-related cue. The hypothesis that butenolide may result from organic matter decay would help explain the germination promotion of the soil seedbank of these South African *Mesembryanthemaceae* and other smoke-stimulated species from non-fire-prone environments, including the desert ephemerals in the present study. Testing of desert soils for the presence of butenolide and a wider screening of butenolide stimulation in non-fire-prone desert species will aid in resolving the true nature of the stimulation of seed germination in these environments.

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