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Original article

Oxygen- and ethylene-induced germination in dormant peach seeds

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Summary

Low embryo viability and recalcitrant germination of peach seeds limits the production of peach seedlings for nursery stock and of new breeding lines. We show that super-ambient oxygen (100% O_2) and, furthermore, co-application of oxygen and ethylene (10 µL L^{-1} oxygen) led to significant germination in seeds from established peach cultivars, including 'Blake', 'Loring', and 'Lovell', and also from selected breeding parents of 'Guardian®' peach. After 42 days in oxygen-ethylene atmosphere germination was close to 100% in 'Blake' and 'Loring' and around 80% in 'Lovell', but was nil in air and around 4 to 20% in air plus ethylene, depending on the cultivar. Germination reached 50% or higher in new breeding lines of 'Guardian®' peach seeds, despite hindrance by fungal infestation. Super-ambient oxygen also accelerated germination of cold-stratified seeds, compared to seeds held in air alone. These data suggest that super-ambient oxygen, particularly in combination with ethylene, might be used as working protocol to achieve substantial germination in dormant peach seeds.

Keywords
dormancy, ethylene, germination, oxygen, peach seed

Significance of this study

What is already known on this subject?

- Poor germination of peach seeds hinders the production of peach seedlings for nursery stock and for new peach breeding lines.

What are the new findings?

- We show that use of oxygen and ethylene leads to robust and uniform germination in dormant peach seeds.

What is the expected impact on horticulture?

- The results are of practical importance for the peach industry, particularly peach breeding programs, which have been struggling with poor seed germination of newly bred germplasm. The results might also be applicable to other stone fruit. We are not aware that this approach has been previously used for peach seed germination.

Introduction

Peach [*Prunus persica* (L.) Batsch] seeds are germinated to propagate seedlings for nursery stocks and to screen for new accessions of germplasm in breeding programs. However, peach seed dormancy, classified as physiological, morphological, physical, and combinational dormancy (Baskin and Baskin, 2004; Finch-Savage and Leubner-Metzger, 2006), is a hindrance to peach seed germination (Martinez-Gomez and Dicenta, 2001). Low seed viability, manifested by low germination rate of only 5% in some years (Thompson, 1998), is also a limitation to the supply of seedling for nursery stock and for new plantings. Low seed viability restricts the availability of newly bred germplasm, because of poor germination of some peach crosses (Tukey, 1933; G.L. Reighard, unpublished data). It is also a limitation to the propagation of valuable germplasm from foreign imports (Thompson, 1998). Seed germination for production of seedlings requires breakage of seed dormancy by cold stratification, a lengthy process predicated on prolonged chilling requirements, particularly for some cultivars (Rodriguez-A and Sherman, 1985; Frisby and Seeley, 1993a, b). An embryo rescue protocol (Tukey, 1933; Fan and Ma, 1993), an alternative method for seedlings production from peach seeds, is labor intensive, inconsistent and results in seedlings which grow abnormally (Frisby and Seeley, 1993b). Hence, progress in peach seed germination would help meet the need of the peach industry and of peach breeding programs.

Previous studies revealed that super-ambient oxygen stimulated germination in dormant seeds from various plant species (Brennan et al., 1978; Esashi et al., 1978, 1982; De Meillon et al., 1990; Gay et al., 1991; Small et al., 1993) and that co-application of oxygen and ethylene further stimulated seed germination (Brennan et al., 1978; Esashi et al., 1982, 1987). Consequently, we examined whether gas regimes consisting of super-ambient oxygen alone as well as oxygen in combination with ethylene might overcome intractable germination in peach seed. We show that the use of this working concept led to onset of significant germination in dormant seeds of established and newly bred peach cultivars.

Materials and methods

Plant material

Peach [*Prunus persica* (L.) Batsch] seeds used for the germination trials outlined below consisted of 'Blake', 'Loring', and 'Lovell' cultivars and from selected lines of 'Guardian®' peach cultivars. 'Lovell' seeds were obtained from Fowler Nurseries, Inc., New Castle, CA, USA. 'Loring' and 'Blake' peach seeds were obtained from fruit grown at the Rutgers horticultural farm in Cream Ridge, NJ, USA. Seeds from select-

ed lines of 'Guardian®' peach fruit, consisting of 5-33-7 and SL-1090 genotypes, were provided by G.L. Reighard, Clemson University, SC, USA. Fruit were harvested when ripe, the pits removed and allowed drying before shelling with Lisa clippers (Imola, Italy) to extract seeds. Only seeds with unbroken integuments were used for the germination trials outlined below.

Germination protocol

Seed handling, germination and stratification studies followed the protocol described by Frisby and Seeley (1993a) with some modifications. Seeds were kept overnight in distilled water and were then held for 20 min in 10% Clorox bleach solution, followed by rinsing for 10 min in sterilized water containing soap (0.25 mL liquid soap L^{-1} water). Seeds were next rinsed for 5 min in sterilized distilled water and were then soaked for few minutes in a fungicide solution consisting of 1 g L^{-1} sterilized water of Cleary 3336 WP (thiophanate methyl) (TreeCareDirect, Minnetonka, MN, USA). Excess solution was drained and the seed allowed drying. Sterilized seeds were placed in a Petri dish, covered with moist heat-sterilized vermiculite and Petri dishes placed in sterilized 6-L sealed jar fitted with a gas inlet and outlet. The jars were then ventilated with different gas regimes consisting of: 1. Air; 2. Air containing 10 µL ethylene L^{-1} gas; 3. Super-ambient oxygen (100% O_2); and 4. Oxygen containing 10 µL ethylene L^{-1} gas. Ventilation with a desired gas mixture was at a rate of 200 mL min^{-1} for 1 h to establish the appropriate gas regime in the head space of the jars. Jars containing seeds in the various gas regimes were held at 24°C for the duration of the experiment, usually for 42 days. When seed were removed periodically to test for germination efficacy in open air, the jars were ventilated again with appropriate gas mixtures, as described above. The entire protocol outlined above was carried out in a laminar hood. To test for effect of various gas regimes on germination, seeds samples were removed from the jars, usually at 7-day intervals and placed on wet filter paper in Petri dish and the latter held in open air at 24°C for 14 days. Seed were considered as having germinated when the emerged radical was several mm long. Samples consisted of 35 seeds in triplicate. Results are expressed as mean of germination percentage for each sampling period ± standard error (SE). Fungal infestation was extensive in non-sterilized germinating seeds but was around 2–3% in sterilized peach seeds from established cultivars, even after 42 days. Sterilized 'Guardian®' seeds, however, were more prone to fungal contamination, a significant hindrance to seed germination, as discussed below.

Stratification

'Lovell' peach seeds, sterilized as outlined above, were placed in humidified low density polyethylene bags and the latter kept in a sealed 6-L jar with a gas inlet and outlet and ventilated with different gas regimes, consisting of a. super-ambient oxygen (100% O_2); b. air (20.9% O_2); or c. sub-ambient oxygen (4% O_2 in nitrogen). The jars, containing sterilized seeds kept in various gas mixtures, were held at 6°C in a Fisher Isotemp refrigerator (Fisher Scientific, Pittsburgh, PA, USA) for cold stratification. To assess the effect of stratification atmospheres on germination efficacy, seeds were removed periodically from the cold, placed on wet filter paper in Petri dish and held in open air at 24°C for 14 days. Seed were considered as having germinated when the emerged radical was several mm long. Seed samples consisted of 35 seeds in triplicate. Results are expressed as mean of germination percentage for each sampling period ± standard error (SE).

Gas regimes

Desired ethylene concentrations in air or oxygen (10 µL ethylene L^{-1} gas) were obtained by bleeding ethylene from a compressed gas cylinder and mixing at appropriate ratios with air or oxygen. Gas mixtures applied to stratified seeds, consisting of air, oxygen (100% O_2) or 4% O_2 (in nitrogen), were delivered from a compressed gas cylinder (AirGas, Piscataway, NJ, USA). To prevent contamination by air-borne microorganisms the various gas mixtures were forced through a sterile microbial filter with 0.45 µ pore size (Fisher Scientific, Pittsburgh, PA, USA), prior to entry onto the jar inlet. Ethylene was purged from applied gases, comprising air or oxygen alone, by passing the gases through a glass tower containing dry potassium permanganate, resulting in <0.1 µL ethylene L^{-1} of the gas efflux. Ethylene concentrations in the various gas mixtures were validated by drawing 1 mL gas samples in 4 replicates from the jar head space using a syringe. Determination of ethylene in syringe-drawn gas samples was by flame ionization Shimadzu GC 8A gas chromatogram mounted with a Poropak Q column, with an

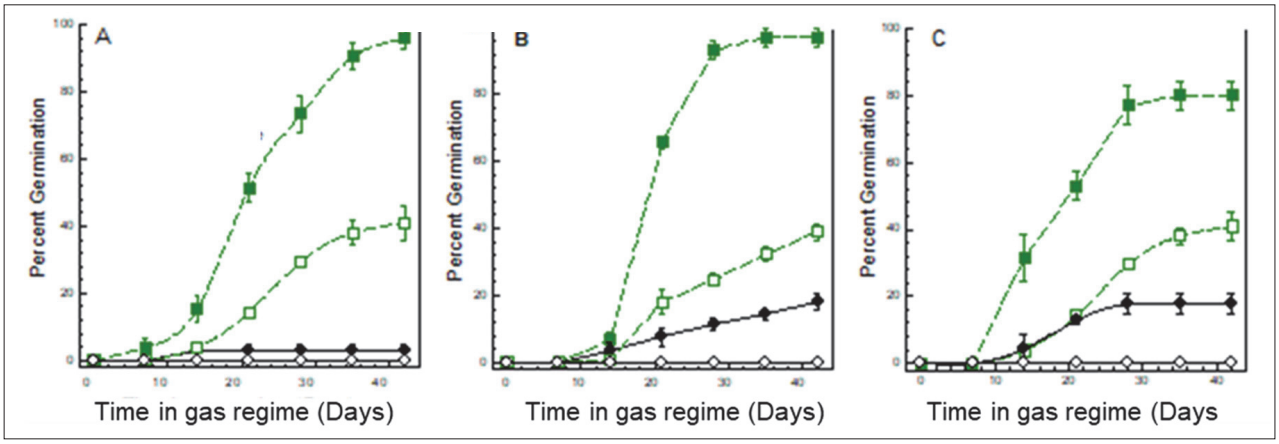


Figure 1. Time-course germination of peach seeds held in air, air plus ethylene, oxygen, and oxygen plus ethylene. Notations are as follows: air (○), air plus ethylene (●), oxygen (□), oxygen plus ethylene (■). Peach seeds held in the various gas regimes were from ripe peach fruit cv. 'Blake' (A), 'Loring' (B) and 'Lovell' (C). The germination protocol is outlined in the Materials and methods section.

injection port and the detector held at 150°C and the column held at 80°C. Ethylene concentration in the test gas samples was gauged against known ethylene standards.

Results

Figure 1 shows time-dependent germination rate of seeds from established peach cultivars, including 'Blake', 'Loring', and 'Lovell', held in air, air plus ethylene, in oxygen or in oxygen plus ethylene. 'Blake' peach seeds (Figure 1A) held in air or air plus ethylene displayed little or no germination over a period of 42 days. In seeds held in oxygen an incipient germination occurred after 14 days, with continued increase in germination up to around 40% by the end of 42 days. In seeds held in oxygen and ethylene, germination occurred already by day 7 and was near completion by the end of 42 days. The results underscore the efficacy of oxygen to initiate robust germination in dormant peach seeds, particularly when combined with ethylene and, by comparison, ineffectiveness to induce germination by air alone or air with ethylene. A similar pattern was observed with regard to the germination of 'Loring' (Figure 1B) or 'Lovell' (Figure 1C); germination for both cultivars was nil in air alone and was below 20% in air plus ethylene. An oxygen atmosphere, however, initiated an upsurge in germination in both 'Loring' and 'Lovell' peach seeds. When combined with ethylene the gas mixture led to almost 100% germination in 'Loring' and around 80% in 'Lovell' by the end of 42 days.

The outlined results were an incentive to examine whether oxygen alone or oxygen plus ethylene might be used to overcome poor germination in breeding lines of 'Guardian®' peach seed (G.L. Reighard, unpublished data). The results show that an oxygen atmosphere stimulated significant germination in 'Guardian®' breeding lines 5-33-7 and SL-1090 after 27 days, amounting to a germination rate of about 25% for cv. 5-33-7 and around 40% for SL-1090. Seeds held in air did not germinate (Figure 2A). Seed germination was around 45% and 55% for genotypes 5-33-7 and SL-1090, respectively, when held in oxygen plus ethylene for the same time duration. Seeds held in air + ethylene did not germinate (Figure 2B). Significant germination of seeds from 'Guardian®' breeding lines highlights the efficacy of the present approach for overcoming intractable germination in peach seeds, particularly when seed germination is very poor in peach breeding lines (Tukey, 1933; G.L. Reighard, unpublished data). However, susceptibility of 'Guardian®' seeds to fungal infestation constrained the efficacy of seeds to respond to the employed gas regimes; early-germinating seeds appear to have escaped interference by infestation but infested seeds were non-viable beyond 27 days and did not germinate. Hence, fungal infestation appears to restrict the germination potential of 'Guardian®' peach seeds and might have been a source of large variation in seed that did germinate (Figure 2).

Cold stratification is used to induce germination in dormant peach seeds but the method requires prolonged exposure to cold temperatures (Rodriguez-A and Sherman, 1985; Frisby and Seeley, 1993a, b). We examined whether release from dormancy in cold-stratified seeds might be accelerated by holding seeds in super-ambient oxygen. Figure 3 shows that the rate of germination of 'Lovell' peach seeds held in air was around 4% after 20 days of cold stratification, around 10% after 40 days and near completion after stratification period of 60 days. By comparison, stratified seeds held in oxygen displayed germination rate of around 18% after 20 days and germination was near completion after 40 days. Conversely, seeds held in sub-ambient oxygen (4% O_2) manifested a germination rate of around 6% even after 60 days of cold stratification. Other studies also show that oxygen deprivation during cold stratification was inhibitory to breakage of dormancy and subsequent germination in seeds from other plant species (Bello et al., 1998; Corbineau et al., 2002). We are not aware of studies making use of super-ambient oxygen during cold stratification to accelerate breakage of

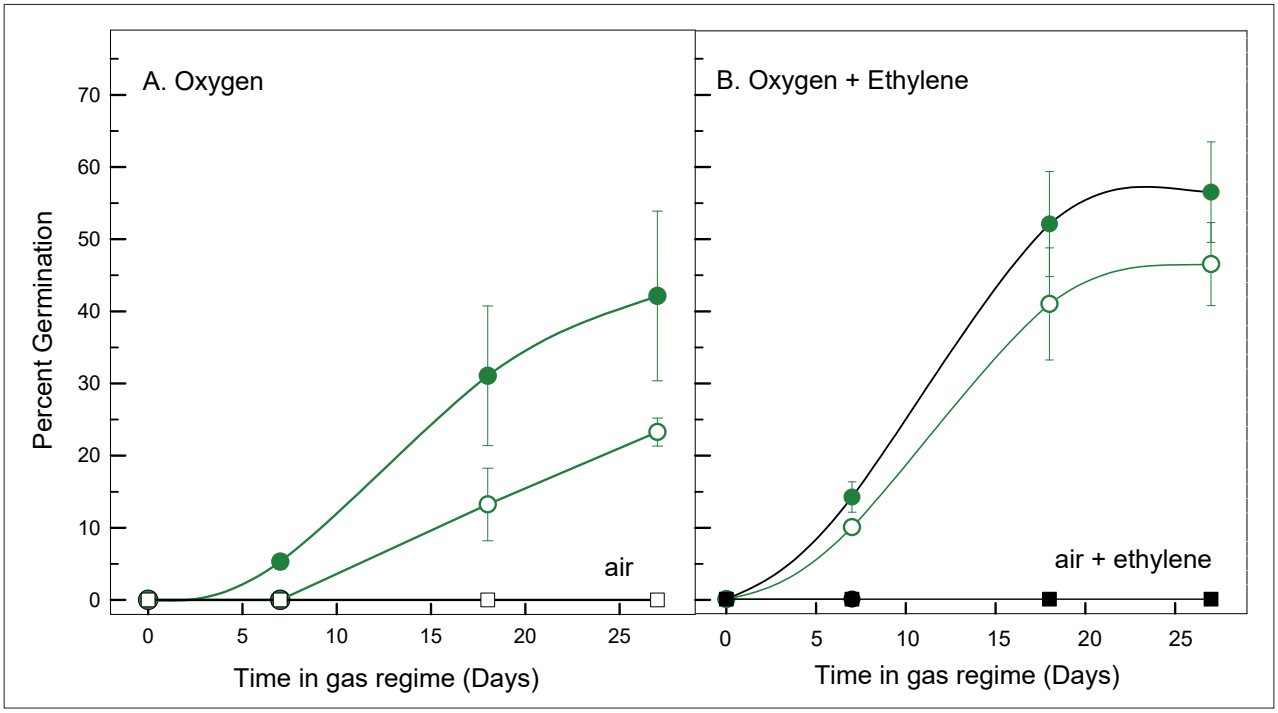


Figure 2. Time-course germination of seeds from cv. 5-33-7 (○) and cv. SL-1090 (●) breeding lines of 'Guardian®' peach seeds held in oxygen (A) and oxygen plus ethylene (B). Seed germination, as influenced by air alone (Figure 2A) or air + ethylene (Figure 2B) is denoted in the body of the graphs. The germination protocol is outlined in the Materials and methods section.

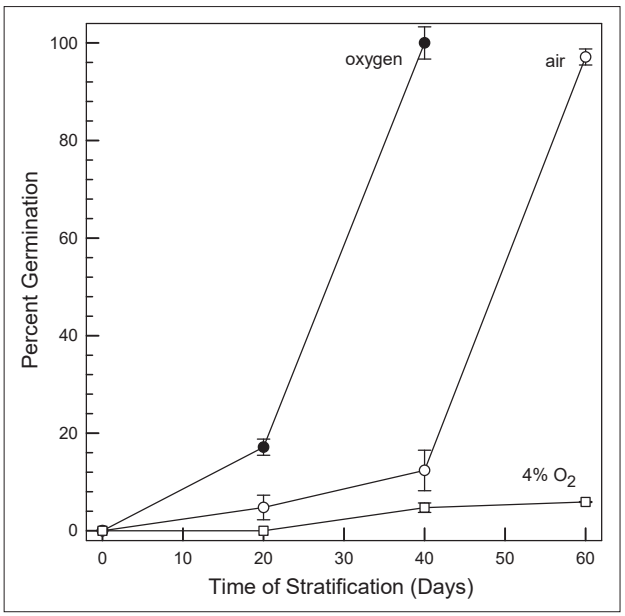


Figure 3. Rate of germination of cold-stratified 'Lovell' peach seeds held at 6°C in air, in super-ambient oxygen, or sub-ambient oxygen. To assess the effect of gas regimes during stratification on subsequent germination stratified seeds were removed periodically and germinated at 24°C for 14 days, as outlined in the Materials and methods section.

seed dormancy, although pressurized air, a mean to increase the oxygen tension, was used to accelerate breakage of dormancy in *Arabidopsis thaliana* seeds (Buijs et al., 2018). The results suggest that oxygen is involved in the release of peach seeds from dormancy during cold stratification, apparently by altering the cellular energy balance and the redox state, as discussed below.

Discussion

It is well established that molecular oxygen plays a role in the breakage of seed dormancy and stimulation of seed germination (Visser, 1954; Poljakoff-Mayber and Evenari, 1958; Roberts, 1962). Moreover, super-ambient oxygen was shown to further stimulate release from dormancy and subsequent germination in seeds from various plant species such as rice (Roberts, 1964), cocklebur (Katoh and Esashi, 1975; Esashi et al., 1987), *Strelitzia juncea* [Narrow-leaved Bird of Paradise] (de Meillon et al., 1990), *Leucadendron daphnoides* (Van Standen and Brown, 1973) and in dormant corn seeds (Violleau et al., 2008). Our results showing that super-ambient oxygen could also overcome poor germination of peach seeds (Figure 1) comply with the above studies. The efficacy of super-ambient oxygen to release peach seed from dormancy is dramatized by oxygen-induced germination in 'Guardian®' breeding lines, which utterly failed to germinate in air or air plus ethylene (Figure 2). The results also underscore the role and potency of oxygen in breakage of peach seeds dormancy during cold stratification; super-ambient oxygen greatly accelerated breakage of dormancy and subsequent germination, compared with seeds held in air (Figure 3). A strong inhibition of germination by sub-ambient oxygen (Figure 3) further emphasize the importance of oxygen in the breakage of seed dormancy and subsequent germination.

Super-ambient oxygen was also applied in combination with ethylene, because this gas mixture was shown to greatly stimulate germination in ragweed (*Ambrosia artemisiifolia*) seeds (Brennan et al., 1978), as well as in dormant seeds of other plant species (Esashi et al., 1978, 1982). Indeed, the results show that oxygen and ethylene stimulated germination, beyond the effect of oxygen alone, leading to near complete germination in established peach cultivars (Figure 1) and resulting in 50% germination or higher in 'Guardian®' breeding lines, which otherwise failed to germinate (Figure 2B). The results are in keeping with other studies showing that oxygen intensified ethylene-induced metabolic and cellular processes. Examples include oxygen- and ethylene-induced respiratory upsurge in potato tuber (Chin and Frenkel, 1976, 1977), initiation of lycopene biosynthesis in a non-ripening *rin* tomato mutant (Frenkel and Garrison, 1976), or enhanced cold acclimation in cucumber seedlings (Frenkel and Callahan, 1997).

An obvious role of oxygen in seed germination is production of metabolic energy by respiration (Roberts, 1964). Accordingly, atmospheric oxygen and super-ambient oxygen in particular were found to increase the energy charge in *Strelitzia juncea* seeds (de Meillon et al., 1990) and in dormant 'Grand Rapids' lettuce (*Lactuca sativa* L.) seeds (Small et al., 1993). Roberts (1964) suggested that the role of oxygen in the release of seeds from dormancy stems also from oxygen-induced changes in cellular redox state, arising apparently from the formation and action of hydrogen peroxide. H_2O_2 and other reactive oxygen species (ROS) were subsequently shown to stimulate release of seeds from dormancy and onset of seed germination (Shaban et al., 2013; Kumar et al., 2015), suggesting that oxygen-induced production of ROS might account for breakage of dormancy and attendant germination of peach seeds. Moreover, heightened ROS production by co-application of oxygen and ethylene (Chin and Frenkel, 1976, 1977) might have been the reason for strong germination of peach seeds, beyond the effect of super-ambient oxygen alone (Figures 1 and 2). ROS production, induced by cold stress (Abdul et al., 2009), may also be a reason for breakage of seed dormancy by cold stratification (Shalimu et al., 2016). Because ROS production is stimulated by high oxygen tensions (Feng et al., 2018), it is conceivable that super-ambient oxygen further stimulated ROS production under cold conditions, accounting for oxygen-induced accelerated germination in cold-stratified peach seeds (Figure 3). ROS are presumed to enhance production of metabolic precursors (Hendricks and Taylorson, 1975), alteration in the composition and function of cellular constituents (Krasuska et al., 2011) such as post-translational modifications of cellular proteins (Arc et al., 2011; Iglesias-Fernandez et al., 2011) or interaction with and alteration of hormonal signaling pathways (Bailly et al., 2008). However, the mode of action of molecular oxygen as well as reactive oxygen species in breakage of seed dormancy awaits further clarification (Mhamdi and Van Breusegem, 2018).

Conclusions

The present results suggest that super-ambient oxygen can be used to accelerate the release of peach seeds from dormancy under room temperature conditions as well as in peach seeds undergoing cold stratification. It appears also that co-application of oxygen and ethylene further stimulate peach seed germination, beyond the effect of oxygen alone. We suggest that these methods might be used as working protocol to achieve germination in dormant peach seeds, particularly from new breeding lines. However, susceptibility of the latter to fungal infestation is a permanent problem (Berjak and Pammenter, 2013), underscored by hindrance to

germination of seeds from 'Guardian®' breeding lines (Figure 2). Nonetheless, the present results are proof-of-principle for the potency of super-ambient oxygen, particularly in combination with ethylene, as a mean for releasing peach seeds from dormancy and onset of strong germination in peach seeds from established or newly selected peach rootstock cultivars. Future studies will examine whether use of this working concept results in peach seedlings with normal growth habit and also whether this protocol might be used to stimulate germination of peach seeds kept enclosed in the pit, thereby simplifying the germination protocol and minimizing adverse fungal infestation.

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