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Effects of garlic genotype on cloves formation under *in vitro* conditions

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Abstract

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Prior to the transfer of multiplicated garlic plants from *in vitro* to *ex vitro* conditions it is necessary to induce the formation of bulbs and to verify the identity of propagated young individuals with their maternal plant. This study deals with effects of exogenous compounds (i.e. paclobutrazol, abscisic acid and ethylene) on formation of bulbs of four garlic cultivars (Lan, Lukan, Emilie, Japo) and on production of ethylene and carbon dioxide (CO_2). In the softneck garlic cv. Emilie, the exogenous application of paclobutrazol increased numbers of daughter cloves and production of both ethylene and CO_2. In the softneck cv. Lukan the formation of cloves was higher after the application of ABA than after the application of paclobutrazol (PP 333). An exogenous application of ethylene inhibited the formation of bulbs. Analyses of DNA polymorphism, performed by means of microsatellite markers, verified the identity of bulbs produced under *in vitro* conditions with their mother plants. The hardneck cv. Lan differed form softneck cvs Lukan, Emilie and Japo.

Keywords: paclobutrazol; abscisic acid; 2-chlorethylphosphonic acid; ethylene; microsatellites

In the Czech Republic, the need of garlic production is still very actual because consumers are not interested in products of a dull taste and a short shelf life (i.e. storability) that are imported from abroad. Under climatic conditions of the Czech Republic, garlic (*Allium sativum* L.), a species belonging to the onion genus *Allium,* does not produce seeds. Flower stems with bulbils and sterile flowers formed by hardneck garlic develop on plants belonging to the group of softneck garlic cultivars only under extreme conditions. The most important problem of Czech garlic production is to obtain sufficient amounts of virus-free planting material of good quality. This problem can be solved by means of vegetative propagation and sanitation of plants under *in vitro* conditions on the basis of a good knowledge of physiological growth reactions of individual cultivars – genetic origin, external conditions, potential propagation *in vitro*, gas production etc.

The first studies on *in vitro* propagation of garlic (*Allium*) plants were focused on effects of different concentrations of N^6-benzyladenine (BA) and 1-naphthyl acetic acid (NAA) in the medium on formation of young plants from flower heads and stem base (Novák, Havel 1981). In cloned, vegetatively propagated leek plants, no morphological and cytological changes were observed and it was possible to transfer and cultivate them under *in vivo* conditions. Another study published by these two authors (Havel, Novák 1988) deals with *in vitro* propagation and the establishment of garlic primary cultures from root segments of 8 garlic cultivars on the Murashige-Skoog medium con-

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taining 2,4-D (2,4-dichlorphenoxyacetic acid) and iP (N^6-(2-isopentenyl)adenine) as well as with possibilities of optimisation of the so-called "regeneration" on the MS medium containing 8.8 µmol of BA and 0.1 µmol of NAA.

Within the framework of physiological studies, the formation of storage organs is supported by lower temperatures, reduced light intensity and increased humidity. In potatoes, the tuberisation is promoted by short-day conditions while in onions this is quite on the contrary, i.e. by long-day conditions. The short-day conditions increase levels of abscisic acid (Biran et al. 1974) and in developing potato stolons the level of growth inhibitors resulting in tuberisation increases (Fišerová et al. 2004). ABA plays an important role in processes of embryo maturation, storage of reserve proteins and seed ripening (Nambara et al. 2010). In vegetative buds, this compound maintains their dormancy and this situation is mentioned by some authors also in garlic (Nagakubo et al. 1993). Sohn et al. (2011) estimated contents of ABA, jasmonic acid and sugar in leaves of growing garlic plants. ABA content increased with the increasing day length and bulb formation; the formation of bulbs was induced by increased contents of jasmonic acid and sugars in leaves.

The first information about the preparation called PP 333 to the end of seventies of the 19th century was published. In this compound, the effective agent is N-dimethylamino succinamic acid, a triazole derivative paclobutrazol (England 1978) with the commercial name CULTAR. It inhibits the biosynthesis of gibberellin, increases stress tolerance of plants, and inhibits processes of senescence (Davis et al. 1991). This means that paclobutrazol can be used in horticulture as a morphoregulator (Hradilík, Fišerová 1986, 1987). The treatment of stem cuttings with PP 333 showed a positive effect on the rhizogenesis (Králík, Šebánek 1989; Davis et al. 1991; Šebánek et al. 1991). There are no data available on its effects on the formation of bulbs.

In plants, different concentrations of ethylene induce a number of biological processes (Burg, Burg 1968; Yang 1980; Macháčková, Ullmann 1987). It influences the structure and properties of plant membranes for example their permeability for some compounds, activates the uptake of ions, and increases secretion of the enzyme α-amylase in kernels of cereals and of cellulase in the abscission zone (Ridge, Amarashige 1984). For example the auxin interacts with ethylene and the application of CEPA stimulates formation of daughter bulbs (Cvikrová et al. 1994).

The strategy of application of explant cultures for propagation of plants (e.g. when eradicating garlic diseases) under *in vitro* conditions requires a verification of genetic variability (and/or identity of produced plants) on the basis of methods of molecular biology used in studies on the polymorphism of DNA. Positive results using microsatellites (Simple Sequence Repeat – SSR) in Okinawan spinach (*Gynura bicolor*) (Liu et al. 2011) and Arabian primrose (*Arnebia hispidissima*) (Phulwaria et al. 2013) were obtained.

The aim of this study was to describe: (1) the effect of growth regulators on increase of bulbs multiplication rate of four cultivars of garlic, (2) the production of ethylene and carbon dioxide as earlier markers of multiplication and growth and (3) the genetic origin of cultivars and to verify the identity between produced bulbs (clones) and donor buds.

MATERIAL AND METHODS

Garlic multiplication and estimation of gaseous markers. Primary garlic tissue cultures were established using apical meristems of garlic cloves (Havel, Novák 1988). Multiplicated garlic plants of the hardneck cv. Lan and softneck cvs Lukan, Emilie and Japo were cultivated on the MS (Murashige, Skoog 1962) medium containing 0.5 mg/l of iP and 0.2 mg/l 1-NAA and supplements paclobutrazol (0.5 mg/l), and/or ABA (0.2 mg/l) or CEPA (1%). One millilitre of 1% Flordimex (active substance is CEPA) was added into a glass vial placed in the cultivation vessel containing the basal MS medium (Fig. 1). In this cultivation vessel, germinating plants released higher concentrations of ethylene from CEPA and showed an exogenous effect of plants cultivated in the aforementioned vessels that were closed with caps enabling the sampling of released gases through a septum. The obtained samples were thereafter analysed by gas chromatography (Fišerová et al. 2001, 2008; Prokeš et al. 2006). Results from 9 repetitions on the cultivar were averaged, mean error calculated and graphically processed by Microsoft Excel 2010.

DNA analysis. Genomic DNA was isolated from young first foliage leaves using the DNeasy Plant Mini Kit (Qiagen, Hilden, Germany). Altogether six

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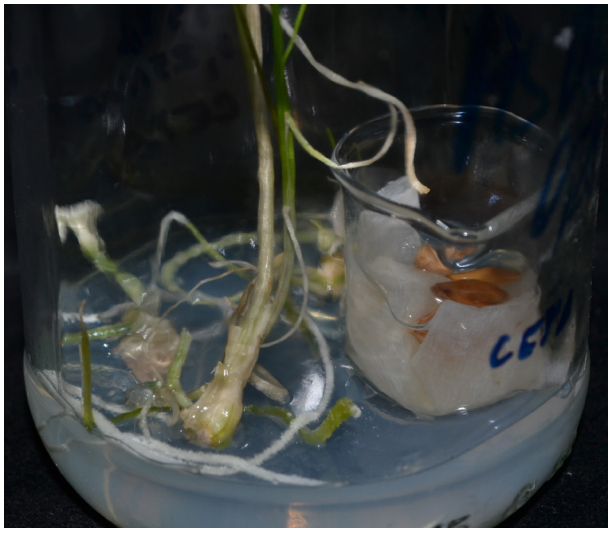

Fig. 1. Bulb formation (cv. Lan on MS medium containing CEPA) – beginning of the experiment

SSR markers (EU909133, EU909134, EU909135, EU909136, EU909137, and EU909139) were analysed. Primer sequences and reaction conditions were used according to Ma et al. (2009). The reaction mixture, electrophoresis by polyacrylamide gel, and visualization with silver staining (0.2% $AgNO_3$) were used according to Vyhnánek et al. (2009). Subsequently, the obtained results were analysed by means of the statistical software FreeTree, version 9.1 (Hampl et al. 2001) using the UPGMA (Unweight Pair Group method with Arithmetic Mean) clustering method and the Jaccard coefficient of similarity (Jaccard 1908). The graphical expression on the matrix was performed using the software TreeView, version 1.6 (Page 1996). The individual statistical indicators (DI – diversity index, PI – probabilities of identity, and PIC – polymorphic information content) for each SSR marker were calculated according to Russell et al. (1997).

RESULTS AND DISCUSSION

Multiplication and gas production

Average number of bulbs produced in cultivation vessels after 7 weeks of cultivation on media containing exogenous growth regulators are presented in Fig. 2. Results obtained in the treatment with the hardneck cv. Lan that formed (similarly as the softneck cv. Japo) 5 to 7 bulbs, after the application of growth regulators were statistically insignificant. The effect of paclobutrazol was statistically significantly manifested in softneck cvs Lukan (Fig. 3a) and Emilie showing symptoms of poor growth (in the control treatment, 1 to 2 bulbs produced 2 to 8 bulbs in the vessel). In well growing garlic cultivars, supplements of ABA (Fig. 3b) and ethylene (Fig. 3c) reduced the formation of bulbs while in case of the poorly growing cv. Lukan the formation of bulbs was significantly increased to 3 pieces. Also Haque et al. (2000) indicates considerable differences in the *in vitro* cloves formation and multiplication factor for some Japanese varieties of garlic but without affecting by growth retardants.

Concentrations of ethylene and CO_2 in cultivation vessels after 7 weeks of growing, young plants are illustrated in Fig 4. In the control treatment, concentrations of ethylene were maximally 50 nl/l; this means that they were still physiologically inefficient (Burg, Burg 1968; Fišerová et al. 2010). As compared with the control, the application of paclobutrazol significantly increased concentrations of both ethylene and CO_2 in the softneck cv. Japo and the formation of bulbs was slightly lower than in control. In the poorly growing cv. Emilie, the formation of bulbs was significantly increased from 2 to 8 per vessel and the concentration of ethylene was increased as well. In case of the softneck cv. Emilie, ABA increased the concentration

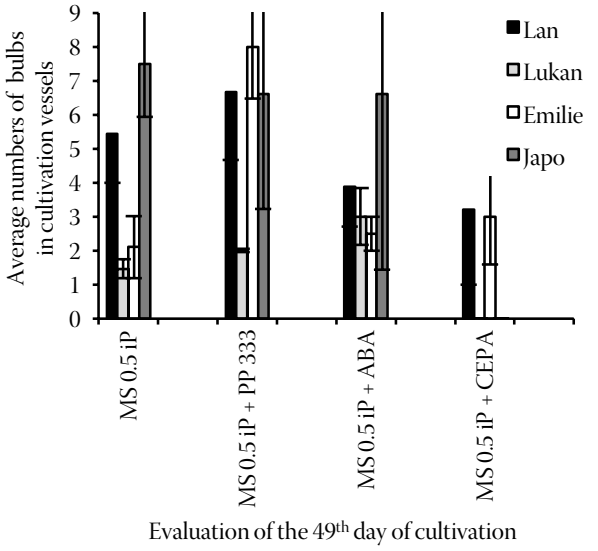

Fig. 2. Average numbers of bulbs in cultivation vessels after the treatment with paclobutrazol (0.5 mg/l), ABA (0.2 mg/l), and CEPA (1%) in garlic cvs Lan, Lukan, Emilie and Japo (after 7 weeks of cultivation)

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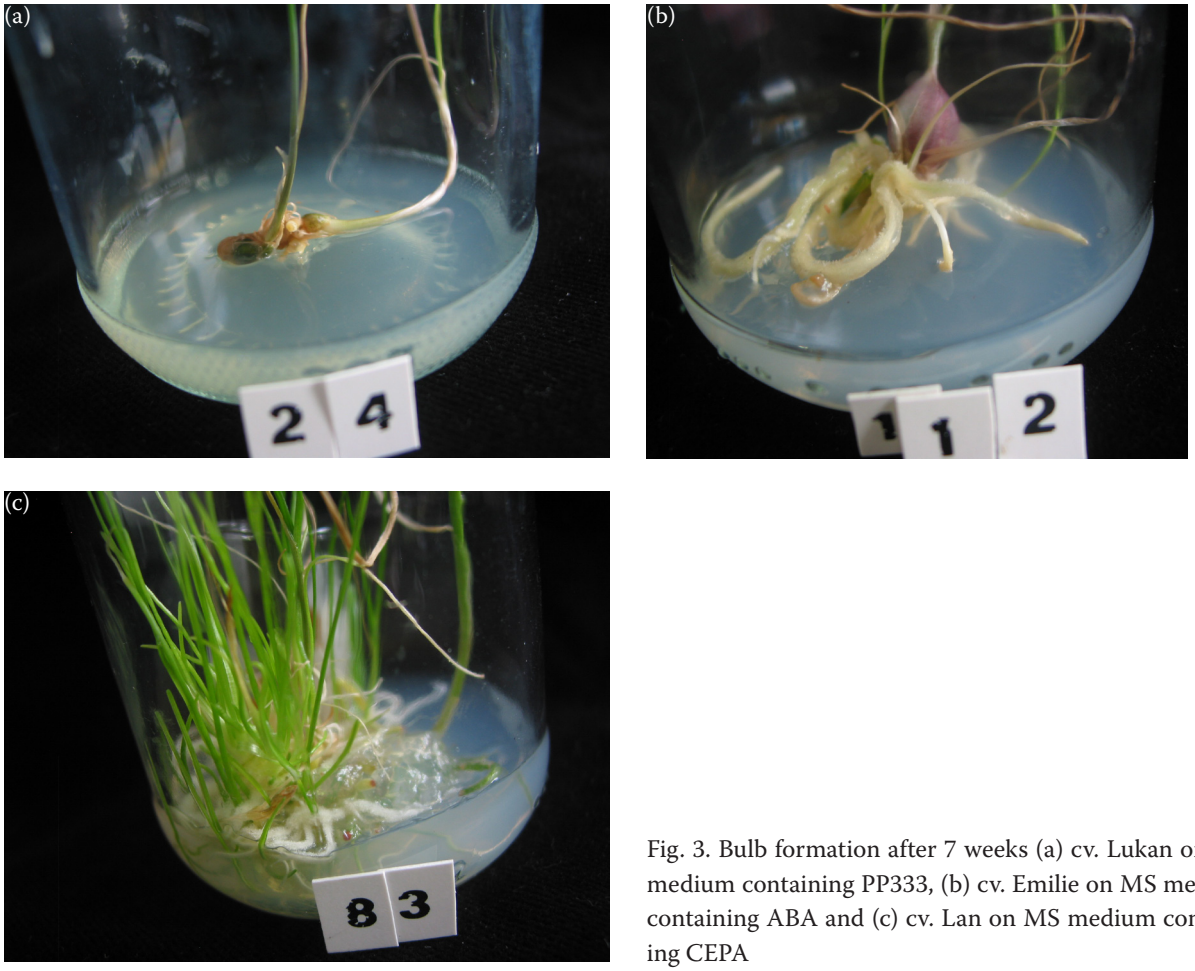

Fig. 3. Bulb formation after 7 weeks (a) cv. Lukan on MS medium containing PP333, (b) cv. Emilie on MS medium containing ABA and (c) cv. Lan on MS medium containing CEPA

of ethylene to the end of the experiment and the concentration of CO_2 was high during the whole experimental period (Fig. 4b). ABA increased the formation of bulbs in the experiment with the cv. Lukan; however, the other cultivars did not respond to the addition of ABA into the medium; they did not produce any new bulbs. An exogenous application of gaseous ethylene resulting in a biological decomposition of CEPA (higher than its physiological effectiveness) caused only an increase in the number of produced bulbs in the poorly regenerating cv. Emilie (Fig. 6). In well growing cvs Lan and Japo, increased concentrations of ethylene caused only their stagnation or even mortification.

SSR markers and identity of regenerants

Due to the need of testing the genetic stability of material cultivated under *in vitro* conditions it was decided to estimate identity in individual cultivars by SSR markers. The resulting values are shown in Table 1. The same markers were used also for the assemblage of the microsatellite panel enabling an identification of Czech garlic cultivars (Ovesná et al. 2014). The size of each PCR product corresponds to the size described by Ma et al. (2009) and Ovesná et al. (2014). These lower values of individual characteristics (12 SSR markers) were caused by lower number of cultivars in our study. De Mattia et al. (2007) described similar findings in the analysis of Sardinia grapevine cultivars.

Garlic varieties have no pedigree because they are derived from plants of the regional origin and were grown spontaneously for many decades. Only the so-called *small* Japo variety is the result of wrong maintenance propagation. The so-called *great* Japo was maintained (thanks to efforts of some growers) in approximately original size. The name Japo II is used for a cultivar that is the result of sanitation of the original *small* Japo by the grower Mr. Kozák. Lukan is one of the oldest Czech varieties. Cv. Lan is the result of the breeding process and selection within the framework of registration tests as a mu-

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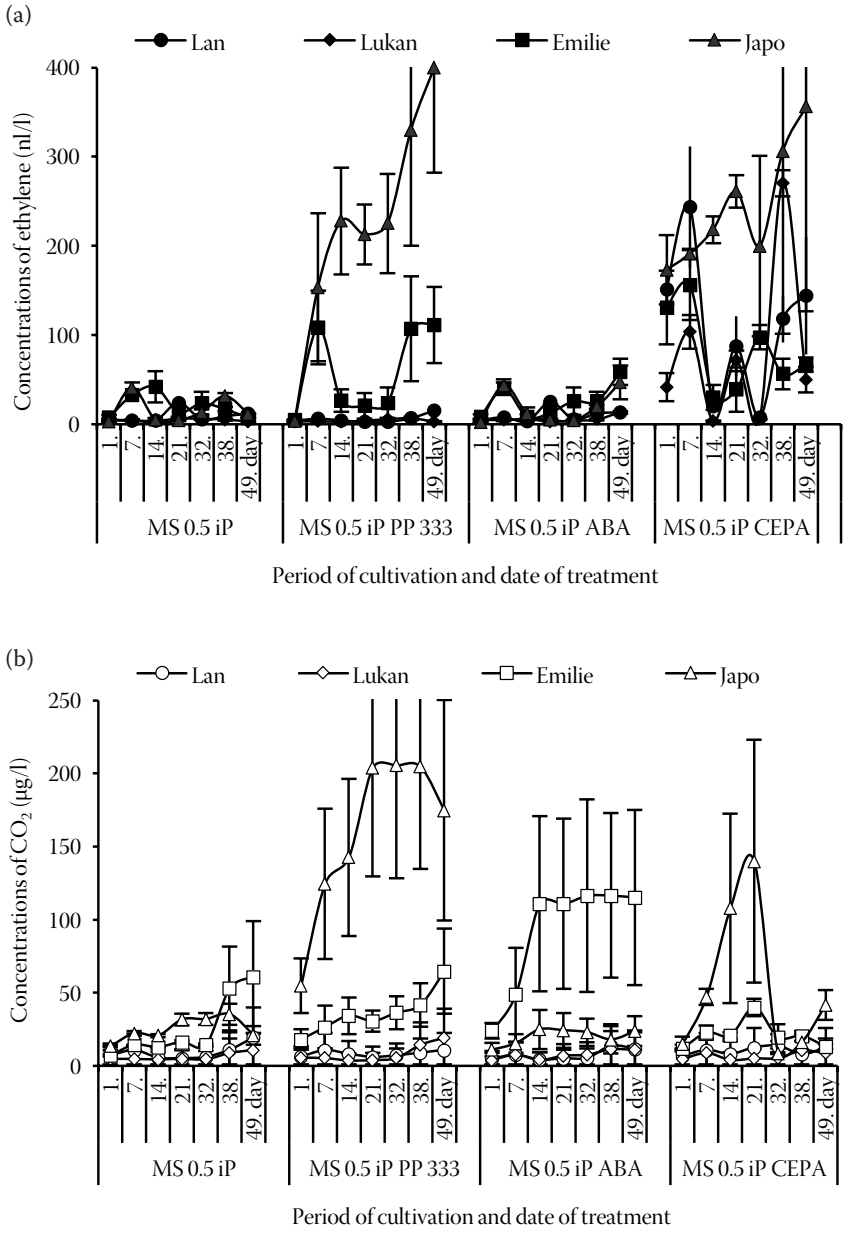

Fig. 4. Concentrations of (a) ethylene (nl/l) and (b) CO_2 in cultivation vessels after 7 weeks of cultivation on the MS medium containing 0.5 mg/l of iP, 0.25 mg/l of NAA and supplements of paclobutrazol (0.5 mg/l), ABA (0.2 mg/l) and CEPA (1%) in garlic cvs Lan, Lukan, Emilie and Japo

Table 1. Characterisation of SSR markers in Czech garlic cultivars and their statistical analysis

Locus name	GeneBank accession No.*	Size range (bp)	N_a	DI	PI	PIC
GB-ASM-040	EU909133	260–300	2	0.49	0.38	0.37
GB-ASM-053	EU909134	160–220	5	0.72	0.05	0.72
GB-ASM-059	EU909135	260–300	4	0.74	0.06	0.72
GB-ASM-072	EU909136	200–280	4	0.62	0.12	0.61
GB-ASM-078	EU909137	180–220	5	0.77	0.04	0.76
GB-ASM-109	EU909139	190–220	4	0.64	0.07	0.63
Mean			4	0.66	0.12	0.63

*SSR – sequence information is available at http://www.ncbi.nlm.nih.gov/; N_a – number of alleles, DI – diversity index; PI – probabilities of identity; PIC – polymorphic information content

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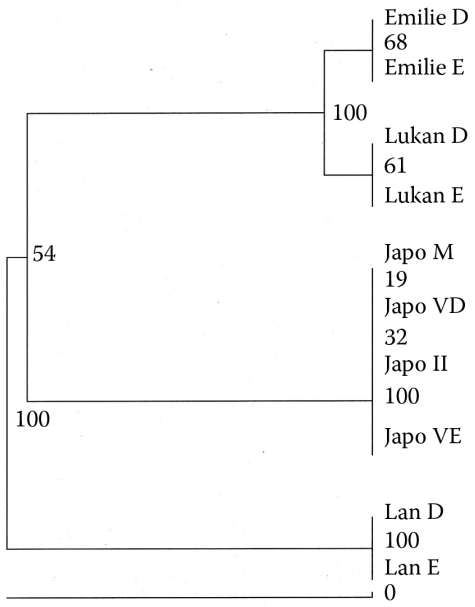

Fig. 5. The dendrogram showing variability and clustering of the analysed genotypes

D – donor plants, E – explants, M – small bulbs, V – big bulbs and II – health improvement

tation of the variety Slavin. Emilie is a new cultivar that originates from an unknown regional variety on the farm of Mr. Kozák and it has not passed through the process of registration, yet.

The dendrogram (Fig. 5) indicates that poorly growing softneck cvs Emilie and Lukan originate from the same genealogical branch. A distant identical origin is indicated also in well growing softneck cv. Japo. As compared with other garlic varieties, the origin of the well growing hardneck cv. Lan (resulting from breeding and selection of plants belonging to the variety Slavin) obviously differs from analysed cultivars mentioned above.

CONCLUSION

This paper presents the origin of garlic cultivars under study. As far as the formation of bulbs in individual garlic cultivars is concerned, it is not important if the plant material belongs to the group of hardneck or softneck varieties but if experimental plants show low regeneration capability (that can be improved by exogenous paclobutrazol) caused by the identic origin of evaluated varieties (as in softneck cvs Lukan and Emilie). In case of poorly growing cultivars, a supplement of ABA into the medium promotes the formation of bulbs while in those that grow well it shows an inhibiting effect. Gases produced by plants in the course of their growth signalise their growth activity. However, if the so-called physiological efficiency of these gases is trespassed, the final result may be an inhibition.

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