Differential and interactive effects of cytoplasmic substitution and seed ageing on submergence stress response in wheat (*Triticum aestivum* L.)

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**ABSTRACT**

Cytoplasmic genomes affect various phenotypes, including abiotic stress responses, through interaction with nuclear genomes in plants. We focused on the effects of cytoplasmic substitution on germination and seedling growth in combinations with submergence and seed ageing, both of which are known to inhibit these traits posing a challenge in agriculture and seed banking. We carried out comparative phenotypic studies of submergence and seed ageing effects using a series of nucleus–cytoplasm (NC) hybrids of wheat, in which 11 heterologous cytoplasms of *Triticum* and *Aegilops* species were combined with a common nucleus. Adopting the test-tube bioassay, germination and seedling growth were studied using aged and non-aged seeds. Imbibed seeds were subjected to 3-days submergence followed by incubation under de-submergence conditions. Seed ageing reduced the germination rates in NC hybrids. Submergence and seed ageing both caused reduction of seedling growth evaluated by shoot length in all or most of the lines. The magnitude of shoot growth inhibition by submergence and seed ageing varied greatly among NC hybrids compared with the nuclear donor, and three distinct response types were recognized. Submergence and seed ageing, in combination, caused leaf chlorosis in most of NC hybrids. Our results suggested that the observed diverse effects of cytoplasmic substitution were exerted through differential interactions with submergence and seed ageing. Further studies are needed to clarify the mechanisms underlying cytoplasmic genome diversity and interaction with nuclear genomes affecting submergence and seed ageing responses in wheat.

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**Introduction**

Nucleus–cytoplasm hybrids (NC hybrids) or alloplasmic hybrids have provided valuable genetic resources to study interspecific and intergeneric diversity of cytoplasmic genomes and NC genome interaction in *Triticum* (wheat) and *Aegilops* (goatgrass) [1]. NC hybrids are produced by replacement of cytoplasms of given paternal nuclear donors by cytoplasms of maternal parents through repeated substitution backcrosses. Kihara [2] proposed NC heterosis in that beneficial and perpetual heterotic effects generated by specific combinations of nuclear and cytoplasmic genomes might be able to improve the agronomic performance in crop plants. In wheat, several studies demonstrated positive and negative effects on disease resistance [3,4], photosynthesis and respiration [5], plant height and heading date [6], yield potential [7], seed quality [8], alterations in transcriptomes and metabolomes [9] as well as abiotic stress tolerance [10,11]. However, practical utilization of cytoplasmic diversity and NC interaction in agriculture remains largely unexplored except for well-studied cytoplasmic male sterility and nuclear restoration of fertility for hybrid wheat production [12–15].

Seed imbibition and germination are pivotal stages of developmental phase transition after seed maturity in flowering plants. High seed viability and vigour leading to uniform and rapid germination is critical for successful seedling establishment [16,17]. Reduction and delay of germination inevitably leads to the loss of seedling vigour and uneven growth particularly under stress conditions. Submergence and seed ageing are known to cause adverse effects on seed germination and seedling growth. Submergence (complete inundation) and waterlogging (saturation of soil with water) caused by floods and heavy rainfalls have become serious threats in wheat production due to global climate changes, leading to 15–20% yield...
loss particularly in Asian countries where rice-wheat rotation is in practice [18-21]. Although genetic variability was reported to exist in submergence tolerance and bread wheat was more tolerant than durum wheat and barley [22], our current knowledge of submergence/waterlogging stress response of wheat is yet quite limited. Seed ageing resulting from prolonged ex-situ storage also poses a challenging concern in seed industry and seed banking [23,24]. In wheat, significant genetic variation was reported in natural and induced seed ageing [25-27]. During imbibition through germination, desiccated orthodox seeds absorb water to initiate a dynamic process of reactivation of complex metabolic machinery, enabling the emergence of radicle and plumule [28]. Rapid and uniform transition from the quiescent to the metabolically active phase is promoted primarily by reactivation of pre-existing mitochondria to supply energy required for subsequent autotrophic seedling growth. Upon imbibition, structurally simple prolamellar bodies rapidly differentiate into fully functional mitochondria [29–34]. This rapid and complex process is controlled both by endogenous and exogenous factors and is vulnerable to disturbances by unfavourable environmental conditions, which lead to slow and uneven germination associated with less vigorous seedling growth and ultimate failure of crop establishment for maximum productivity [16,35].

We studied the extent and magnitude of effects of cytoplasmic substitution and seed ageing on submergence stress response in wheat. We compared between aged and non-aged seeds of NC hybrids possessing eleven different cytoplasms of Triticum and Aegilops species in a common nuclear background of wheat. We herein report our results showing diverse and interactive effects of these stresses on germination and early seedling growth.

### Materials and methods

#### Plant materials

Eleven lines of NC hybrids used were produced by combining cytoplasms of Triticum boeoticum and 10 Aegilops species with a nucleus of wheat (Triticum aestivum) cultivar Chinese Spring (hereafter abbreviated as CS) by repeated substitution backcrosses using CS as a recurrent paternal parent (Table 1). Original NC hybrids were provided in 1988 by K. Tsunewaki, now Professor Emeritus of Kyoto University, and were maintained by backcrosses and/or self-pollination. Aged and non-aged seeds of both NC hybrids and CS were used in bioassay for seed germination and seedling growth. Aged seeds were all harvested in 1997 and non-aged seeds in 2016. All seeds were stored in plastic containers with silica gel at 4 °C in a refrigerator until use.

#### Bioassay method for assessing seed germination and seedling growth

A test-tube method developed and successfully used in rice [36–39] was adopted to study seed germination and seedling growth. Aged and non-aged seeds of NC hybrids and CS were imbibed individually for 2 days in glass test-tubes (inner diameter of 14 mm, height of 165 mm) filled with 15 mL of deionized water (10 cm in depth). Submergence stress was given to imbibed seeds by further incubation under water for 3 days. After imbibition with or without submergence, water was drained out and imbibed seeds were kept at the bottom of test-tubes with the embryo side up and incubated under de-submergence conditions. Incubation conditions of seeds and seedlings were adjusted at day/night temperatures of 15 °C/10 °C with a photoperiod of 12 h light and 12 h dark under LED lamps at a light-intensity of ca. 120 μmol m⁻² s⁻¹ in a walk-in incubator. Seed germination was judged based on protrusion of a radicle with two seminal roots and a coleoptile length longer than 15 mm. Fifteen seeds of about the same size were used in each bioassay and each assay was repeated more than three times in all lines.

The effects of cytoplasm substitution, submergence and seed ageing on seedling growth were evaluated based on the variables of non-aged seeds and $d^{AG}$ of aged seeds, which denoted shoot length at the

| Code   | Cytoplasm donor          | Plasmon | Nuclear genome |
|--------|--------------------------|---------|----------------|
| C01    | T. boeoticum* aegilopoides | A       | A              |
| C03    | Ae. umbellulata           | U       | U              |
| C04    | Ae. squarrosa² typica     | D       | D              |
| C07    | Ae. uniaristata           | N       | N              |
| C08    | Ae. speltoides ligustica  | S       | S              |
| C10    | Ae. sharonensis           | $S^i$   | $S^i$          |
| C12    | Ae. bicornis              | $S^o$   | $S^a$          |
| C13    | Ae. mutica²               | T       | T              |
| C17    | Ae. speltoides aucheri²   | S       | S              |
| C18    | Ae. searsii               | $S^a$   | $S^a$          |
| C31    | Ae. ovata³               | M⁰       | MU             |
| C35    | Ae. crassa²              | D²       | DM             |

Table 1. List of NC hybrids and their common nuclear donor wheat.

CS T. aestivum cv. Chinese Spring B ABD

Synonyms: "T. monococcum ssp. aegilopoides," "Ae. tauschii," "Amblyopyrum muticum," "Ae. speltoides ssp. speltoides," "Ae. geniculata." C01 shows a considerable level of self-fertility but with low plant vigour, and most likely a spontaneous revertant of line C01 with A-type cytoplasm from T. boeoticum (unpublished).
13th day of incubation without submergence. The variables \( b \) of non-aged seeds and \( b_{AG} \) of aged seeds denoted shoot length after 3-days submergence given to 2-days imbibed seeds followed by incubation under de-submergence for additional 10 days. The total length of incubation after imbibition was thus 13 days for seedlings both with and without submergence.

The ageing effects on shoot length inhibition by submergence were measured by \( \mu(b_{a} - a) - (\mu a_{AG} - b_{AG}) \).

### Statistical analyses

Germination rate was analyzed by Mann–Whitney \( U \) test using the R package 'exactRankTests' [40]. Three-way analysis of variance tests were conducted based on the four variables \( a, b, a_{AG} \) and \( b_{AG} \) to examine the effects of cytoplasmic substitution, submergence,
seed ageing and their interactions on seed germination and seedling growth. Mean comparisons of the four variables in each of CS and NC hybrids were made according to Steel-Dwass test using the software R package 'EZR' [41]. Significant differences in shoot length inhibition with and without submergence between aged and non-aged seeds ($\mu_{a-b^{AG}}$ vs $\mu_{b-b^{AG}}$) were determined by Mann–Whiney $U$ test. Pair-wise comparison of ageing effects on shoot length inhibition by submergence measured by $\mu(\mu_{a-b})-(\mu_{a^{AG}-b^{AG}})$ was made according to Steel test using 'EZR' [41].

**Results and discussion**

**Effects of cytoplasm substitution, submergence and seed ageing on germination**

Phenotypic assay is critical for precise evaluation of complex traits of interest. A pilot experiment examining the sensitivity of wheat seeds and seedlings to submergence showed that both seed germination and seedling growth were completely arrested under continuous submergence. We adopted a simple and rapid test-tube bioassay developed in rice [36] with modifications for wheat. We first examined the effects of duration of imbibition for germination and subsequent seedling growth in test-tubes and found that an optimal period of imbibition was 2 days. The germination rate (%) after 2-days imbibition followed by de-submergence was compared among CS and 11 lines of NC hybrids (Table 1) using aged and non-aged seeds. Without submergence, NC hybrids of C04, C08, C13 and C31 showed significantly decreased germination (Figure 1). With 3-days submergence, although no statistical differences were found between aged and non-aged seeds in all lines, the pooled data showed significantly lower germination rates in aged seeds than in non-aged seeds both with and without submergence. The three-way analysis of variance test confirmed significant effects of cytoplasmic substitution, submergence and seed ageing as well as their interactions on germination, except for the two-way interaction of lines vs. submergence (Table 2).
Effects of cytoplasm substitution, submergence and seed ageing on seedling growth

Both submergence and seed ageing are known to reduce germination and seedling growth in wheat [25–27]. We evaluated the effects of heterologous cytoplasms in combination with submergence and seed ageing on seedling growth using CS and NC hybrids. Imbibed seeds were incubated under de-submerged conditions either for 10 days after 3-days submergence or for 13 days without submergence. Shoot growth was evaluated based on the shoot length of seedlings from aged and non-aged seeds. The variables $a$ and $a^{AG}$ represent shoot length of seedlings derived from non-aged and aged seeds, respectively, at the 13th day of incubation of 2-days imbibed seeds without submergence. Variables $b$ and $b^{AG}$ represent shoot length of seedlings derived from non-aged and aged seeds, respectively, at the 10th day of incubation of 2-days imbibed seeds with 3-days submergence. Significance of mean differences in the four variables was determined according to Steel-Dwass test using a software R package 'EZR' [41].

Effects of cytoplasm substitution, submergence and seed ageing on seedling growth

Both submergence and seed ageing are known to reduce germination and seedling growth in wheat [25–27]. We evaluated the effects of heterologous cytoplasms in combination with submergence and seed ageing on seedling growth using CS and NC hybrids. Imbibed seeds were incubated under de-submerged conditions either for 10 days after 3-days submergence or for 13 days without submergence. Shoot growth was evaluated based on the shoot length of seedlings from aged and non-aged seeds. The variables $a$ and $b$ denoted the measured shoot length of seedlings from non-aged seeds without and with submergence, respectively, whereas $a^{AG}$ and $b^{AG}$ respectively denoted those of seedlings from aged seeds without and with submergence. Cytoplasmic substitution, submergence and seed ageing were all inhibitory in most of the lines, but large variabilities were observed in their effects (Figure 2). Pairwise comparisons with CS showed that shoot length decreased in all NC hybrids except for C07 (N), which showed an increase in shoot length. With submergence, all seedlings of NC hybrids from non-aged and aged seeds showed decreased shoot lengths, but the seedlings of C13 (T) from non-aged seeds and those of C07 (N) from aged seeds did not show any changes. These observations suggested that the T-cytoplasm of Ae. mutica in C13 conferred an equivalent level of submergence tolerance on seedlings from non-aged seeds to that of CS, while the N-cytoplasm of Ae. uniaristata in C07 conferred an equivalent or even increased level of submergence tolerance on seedlings from aged seeds. The three-way analysis of variance test using the four variables revealed significant effects of cytoplasmic substitution, submergence, seed ageing and their interactions (Table 2).

We next compared the response patterns to submergence and seed ageing of CS and NC hybrids. Three distinct groups with different response patterns were recognized among the lines. The first group I consisted of CS (B), C13 (T) and C31 (M9), in which the shoot length decreased in an order of $a > b > a^{AG} > b^{AG}$ (Figure 3(a)). In CS (B) and C31 (M9), no differences were observed between $b$ and $a^{AG}$, but in C13 (T) $b$ was greater than $a^{AG}$. In this group, inhibition of shoot length by seed ageing without

![Figure 3](image-url)
submergence measured by the derivative variable of $\mu_a$-$\alpha^{AG}$ was larger than that with submergence measured by $\mu_b$-$\beta^{AG}$ (Figure 3(b)). The second group II consisted of seven NC hybrids of C01mf (A), C04 (D), C08 (S), C10 (S'), C12 (S''), C18 (S'') and C35 (D'), in which the shoot length decreased in an order of $a > a^{AG} > b$ (Figure 4(a)). Among this group, $\mu_a - a^{AG}$ was larger than $\mu_b - b^{AG}$ in C01mf (A), C04 (D) and C35 (D'), while no differences were observed in C08 (S), C10 (S'), C12 (S''), C18 (S'') (Figure 4(b)). The third group III consisting of C03 (U) and C07 (N) showed shoot length in an order of $a = a^{AG} > b$ (Figure 5(a)), C03 (U) showed a greater value of $b$ than $b^{AG}$ (Figure 5(a)) and a smaller $\mu_a - a^{AG}$ than $\mu_b$-$b^{AG}$ (Figure 5(b)). On the other hand, C07 showed equivalent values of $a$ and $a^{AG}$ as well as $b$ and $b^{AG}$ (Figure 5(a)), suggesting no ageing effects on shoot inhibition by submergence. No significant differences were found between $\mu_a$-$\alpha^{AG}$ and $\mu_b$-$\beta^{AG}$ in this NC hybrid (Figure 5(b)).

**Interactive effects of cytoplasmic substitution, submergence and seed ageing on seedling growth**

The effects of seed ageing on shoot length inhibition by submergence were further studied using a derivative variable $\mu(\mu_a$-$b$)-( $\mu a^{AG}$-$b^{AG}$). All lines except for C03 (U) showed positive values, which indicated that the magnitude of inhibition by submergence was greater in non-aged seeds than in aged seeds (Figure 6). C03 was unique showing greater inhibition by submergence in aged seeds (Figure 5(b)). A majority of NC hybrids showed smaller values of $\mu(\mu_a$-$b$)-( $\mu a^{AG}$-$b^{AG}$), but C04 (D), C13 (T) and C31 (M') showed no differences compared to CS. The results suggested that in these NC hybrids a combined effect of
submergence and seed ageing was equivalent to the effect of submergence in non-aged seeds. Although the reason remains unknown, C01mf (A) showed an even higher value of $l(a-bAG-bAG)$. The significant interactive effects of cytoplasm substitution, submergence and seed ageing were verified by three-way analysis of variance test (Table 2). The observed changes in the effects of submergence and seed ageing in different NC hybrids indicated differential interaction between these two stresses with heterologous cytoplasms.

It was notable that seed ageing caused frequent chlorosis (Figure 7) of seedlings grown from submerged seeds in all NC hybrids used except for C07 (N). Submergence per se did not cause chlorosis. It was notable that C07 (N) exhibited no ageing effects.
on shoot length inhibition by submergence (Figure 5). More severe chlorosis occurred in seedlings from aged seeds submerged for a longer period of time (9-days in Figure 7(b)). The observation suggested that a combination of cytoplasm substitution, submergence and seed ageing disrupted development of functional chloroplasts in wheat seedlings.

**Cytoplasmic diversity affecting stress responses**

Cytoplasmic genomes play important roles in stress tolerance, signalling and adaptation through interaction with nuclear genomes in plants [42–46]. Studies of Arabidopsis thaliana revealed intraspecific diversity of cytoplasms and nucleus–cytoplasm interactions affecting a wide range of plant phenotypes. Using reciprocal F2 families and recombinant inbred lines, significant variations were shown in their cytoplasmic genomes and nucleus–cytoplasm interactions that greatly affected natural variations in germination capacity and metabolism [47,48]. Nucleus-cytoplasm interaction affecting various adaptive phenotypes in the field also was reported in a study of cytolines (synonymous with NC hybrids) produced by diallele crosses among natural accessions [49]. In wheat, post-anthesis heat tolerance increased in seven lines of NC hybrids possessing cytoplasms of Aegilops species based on chlorophyll content and quantum efficiency of photosystem II [10]. T-type cytoplasm of Ae. mutica causing a delay of heading modified the magnitude of QTL controlling dry matter weight in wheat genetic backgrounds [50]. A study of wheat NC hybrids possessing a cytoplasm of Hordeum chilense, a wild relative of barely, showed altered transcriptomes and metabolomes [9].

In our previous study, we demonstrated marked diversities affecting submergence stress response in both cytoplasmic and nuclear genomes in wheat using a series of NC hybrids and wheat accessions [51]. In the present study, we evaluated wheat response to submergence in combination with seed ageing. Phenotypic assessment of seed germination (Figure 1) and seedling growth (Figures 2–6) with and without submergence using aged seeds and non-aged seeds demonstrated differential interaction of cytoplasm substitution, submergence and seed ageing (Table 2). Submergence inhibited seedling growth in CS and most NC hybrids, and seed ageing inhibited both germination and seedling growth, but their effects were not additive and heterologous cytoplasms either increased or even alleviated the deleterious effects of these stresses. Particularly, the T-cytoplasm of Ae. mutica in C13 conferred an equivalent level of submergence tolerance on seedlings from non-aged seeds to that of CS, and the N-cytoplasm of Ae. uniaristata in C07 conferred either an equivalent or even increased level of submergence tolerance. Taken together, our results showed that cytoplasmic substitution and seed ageing gave differential effects on submergence stress response among NC hybrids. It is emphasized that the use of rejuvenated seeds is important in studying complex stress responses such as submergence.

The adverse effects of submergence are primarily attributed to reactive oxygen species produced under anoxia and hypoxia, which in turn are associated with oxidative stress upon re-oxygenation during de-submergence [52–54]. Reactive oxygen species are also produced and accumulated in mitochondria and chloroplasts and damage seeds during ageing to affect the progression of imbibition and germination [53–59]. It has been reported that genebank seed samples of

\[ \text{(A) 2di+3ds+10dg} \]

\[ \text{(B) 2di+9ds+10dg} \]
wheat stored for 23–33 years at 4°C showed chlorophyll deficiency during early seedling growth [27]. We observed that seed ageing in combination with submergence caused frequent chlorosis (Figure 7) of seedlings of all NC hybrids used except for C07 with N-cytoplasm of *Ae. uniaristata*. Further study is needed to clarify the involvement of organellar genomes and their interaction with nuclear genomes in their responses to submergence and seed ageing.

**Conclusions**

Comparative phenotypic assessment was made of germination and early seedling growth using non-aged and aged seeds with and without submergence stress in the nucleus–cytoplasm hybrids possessing heterologous cytoplasms of *Aegilops* species with a common nuclear background of wheat. These heterologous cytoplasms affected seed germination and seedling growth through differential interaction of submergence and seed ageing. Our results point to the necessity of further study of cytoplasmic genetic diversity and nucleus–cytoplasm interaction affecting submergence, seed ageing and other adaptive traits with agronomic importance.

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**Disclosure statement**

All authors declare that they have no conflict of interest; they are entitled to the authorship and have approved the final version of the manuscript.

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